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CARDIOPROTECTION BY ACTIVATION OF THE NO/cGMP PATHWAY, A BLOOD REPERFUSION MODEL

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled CARDIOPROTECTION BY ACTIVATION OF THE NO/cGMP PATHWAY, A BLOOD REPERFUSION MODEL submitted by RODERICK MACARTHUR in partial fulfillment of the requirements for the degree of MASTER OF SCIENCE IN EXPERIMENTAL SURGERY.

Abstract

Sodium nitroprusside (SNP) enhances the recovery of left ventricular function during crystalloid reperfusion (CR) of hearts subjected to cardioplegic arrest and prolonged storage by a nitric oxide/cyclic guanosine monophosphate (NO/cGMP)-mediated mechanism. This study evaluates the cardioprotective potential of SNP with blood reperfusion (BLR).

All hearts were arrested and stored (5-8 hrs, 4^0C). Two groups of experiments were performed: hearts receiving either CR or BLR with the use of a heterotopic rat heart transplantation model. Hearts were reperfused either in the presence or absence of SNP.

SNP improved recovery of function in the CR group (100%). Functional recovery in the BLR-untreated group was poor (23%). While SNP increased cGMP content in BLR hearts ($p<0.05$) at the start of reperfusion, it did not enhance functional recovery (22%). SNP protects hearts subjected to arrest, storage and CR, however, the efficacy of SNP-mediated cardioprotection with BLR is questioned.

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Introduction

Overview

Cardiomyocytes and endothelial cells suffer a myriad of reversible and irreversible injuries from ischemia-reperfusion (IR) during cardiac surgery. Surgeons have minimized the extent of cellular injury and necrosis by using cardioplegia, and modifications to the composition and methods of delivery of cardioplegic solutions during cardiac surgery have contributed to improved patient outcome.[1] Although the mechanisms which underlie IR injury are not completely understood, recent evidence suggests that nitric oxide (NO) may be important in mediating both protection as well as injury in the myocyte. The cardioprotective effects of NO and the consequences of augmenting NO production have been extensively studied.[2-12] L-arginine supplementation and the administration of exogenous NO (via an NO donor) is thought to protect the myocardium from IR injury through 4 general mechanisms:

1. Blocking of neutrophil infiltration and adherence[13]
2. Scavenging of oxygen-derived free radicals[13]
3. Vasodilating directly the coronary circulation[14]
4. Decreasing oxygen demands through vasodilatation-induced hypotension[14]

It is clear that the administration of NO precursors, NO donors or nitric oxide synthase (NOS) inhibitors in the setting of IR may also be detrimental. NO may exacerbate cardiac IR injury by combining with superoxide to produce peroxynitrite ($ONOO^-$) and

oxygen free-radicals.[15-17] The focus of the current study is to assess the mechanical, biochemical and metabolic consequences of cardiac IR in an *in vivo* blood reperfusion model which closely correlates with the clinical scenario of cardiac transplantation. We hypothesize that in such a model NO may be protective by altering glucose metabolism, by scavenging of oxygen free-radicals (OFRs) and by altering neutrophil infiltration and adherence.

Biology of Nitric Oxide

It was suggested in 1987 that endothelium-derived relaxing factor (EDRF) was simply NO.[18-20] Palmer *et al.*[21] showed that NO is formed endogenously in endothelial cells from L-arginine. This discovery opened up a new era of biological and physiological research. It is now recognized that NO has major roles in smooth muscle relaxation, platelet inhibition, neurotransmission, hormonal release and immune function.[22]

NO has been implicated in a wide range of physiological roles in the cardiovascular system.[23] These include:

1. Regulation of vascular tone
2. Regulation of myocardial contractility
3. Antithrombotic effects in the vasculature
4. Regulation of endothelial-neutrophil interactions
5. Regulation of endothelial integrity and permeability
6. Regulation of vascular cell proliferation

Dysregulation of NO production may play a role in the pathogenesis of several cardiovascular disorders including essential hypertension, reperfusion injury, atherosclerosis and myocardial depression associated with septic shock.[24-27]

Biochemistry of Nitric Oxide Synthesis

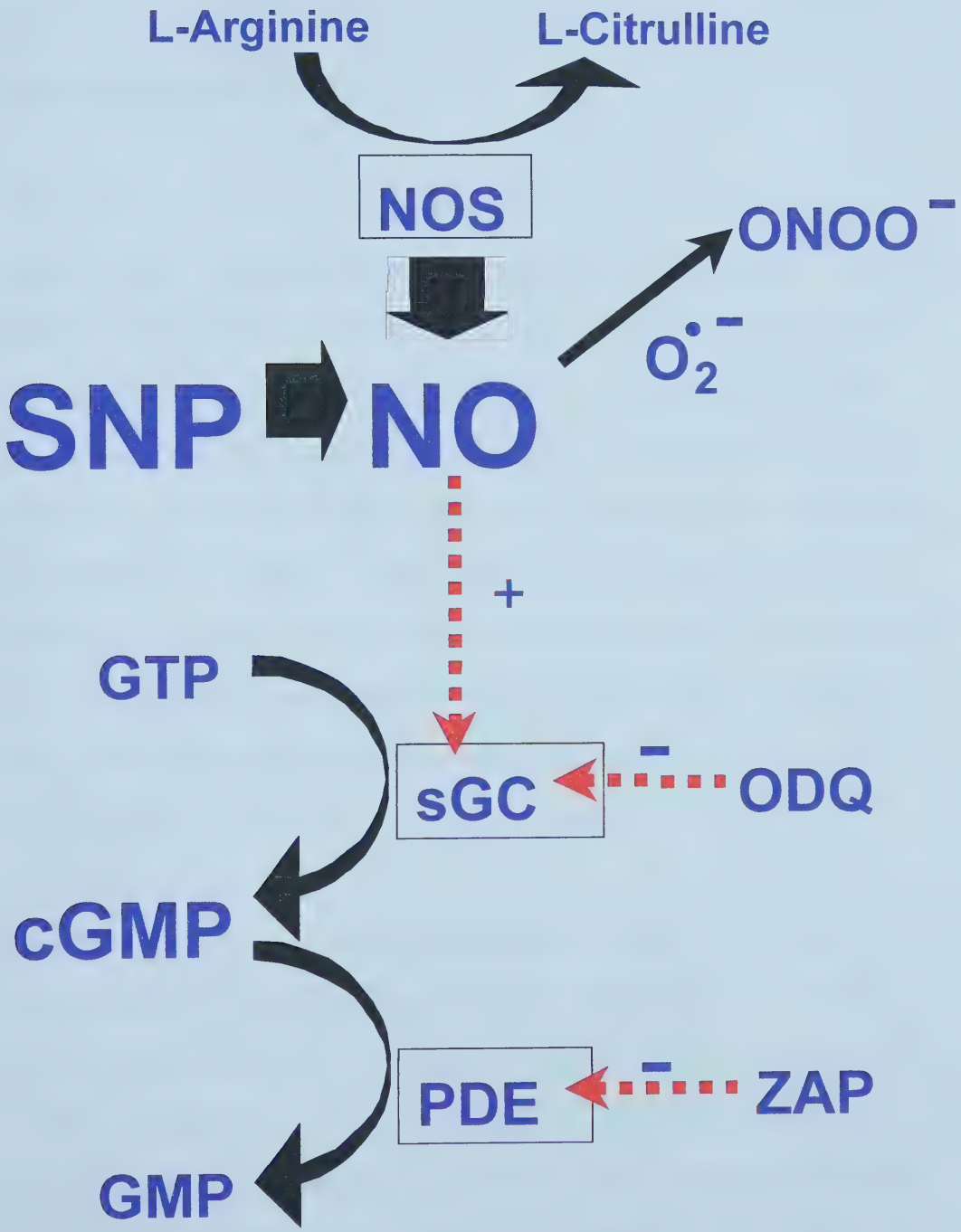
L-arginine is the precursor for NO synthesis by the vascular endothelium.[28] The formation of NO is catalyzed by the enzyme nitric oxide synthase (NOS) which converts L-arginine to L-citrulline (figure 1).

L-ARGININE + nNADPH + mO_2 ➔ L-CITRULLINE + NO + $nNADP^+$ [29,30,38-40]

NO production has also been shown to occur within cardiac myocytes where various isoforms of NOS are present.[31-33] Although L-arginine is an NO "precursor", there are several other potential NO "donors" (e.g., sodium nitroprusside) which release NO under physiologic conditions and yield NO without the need for metabolic conversion by NOS.[28,34]

NO is a small gaseous paramagnetic radical that is weakly soluble in water but readily soluble in lipid media.[2,18,36] These properties allow it to diffuse readily across biological membranes and account for its rapid diffusion out of the circulation and into cells or circulating components of the blood where it can exert its effects.[18,30] NO is

Figure 1. Nitric Oxide Synthesis and Transduction Pathways



continuously released during baseline conditions and NO synthesis is further enhanced by physical stimuli such as shear stress, pulsatile stretch and hypoxia.[37]

Isoforms of Nitric Oxide Synthase

There are two main isoforms of NOS. One is a constitutive enzyme (cNOS) that is present in the vascular endothelium (eNOS), neuronal cells (nNOS) and several other cell types.[23] Constitutive NOS is regulated by Ca^{2+} and calmodulin and produces modest amounts of NO in the presence of sufficient Ca^{2+}.[41-43] It follows that in the vascular endothelium, cNOS plays a major role in maintaining basal vascular tone.[44] The other main isoform of NOS is an inducible enzyme (iNOS). Inducible NOS has been found in macrophages and neutrophils.[45,46] This isoenzyme is immunologically activated by exposure to bacterial endotoxin and various endogenous cytokines.[47-49] Inducible NOS activity is regulated at the transcription level and is not affected by intracellular Ca^{2+} levels.[50] Once induced, iNOS is capable of generating far greater quantities of NO per mole of enzyme than cNOS.[51,52]

The different cell types comprising cardiac muscle express one or more of the three isoforms of NOS. Neuronal NOS is expressed in sympathetic nerve terminals and regulates catecholamine release by the heart. Endothelial NOS affects contractile tone and inhibits vascular smooth muscle cell proliferation. This isoform is also known to inhibit platelet aggregation and neutrophil adhesion, promote diastolic relaxation and decrease oxygen consumption. Inducible NOS gene transcription and protein expression

are increased in all myocardial cell types after exposure to a variety of inflammatory cytokines. Given the multiplicity of NOS isoforms expressed in cardiac muscle and the many potential targets for the NO produced, tight molecular regulation of NOS expression and activity appear to be necessary to coordinate the many roles of NO in heart function, in health and disease.[136]

NO has the potential to possess both beneficial and detrimental effects on cardiac function. Low concentrations of NO (generated from either eNOS or iNOS) have several beneficial effects, whereas high NO concentrations (generated only from iNOS) are generally associated with cardiotoxicity.[48-50] The positive effects are believed to occur secondary to increased blood flow and improved ventricular relaxation secondary to cGMP augmentation. It is thought that the negative effects occur as a result of an interaction with superoxide anion, producing $ONOO^-$. Since iNOS activity can increase NO production in an unregulated manner which is not Ca^{2+}-dependent, induction of this isoform is typically blamed for $ONOO^-$ production and cell death.[48] Further studies are necessary to define the optimal conditions in which NO acts to enhance the recovery of myocardial function after an ischemic insult.[53]

Nitric Oxide and cGMP

NO performs a variety of functions including endothelium dependent vasorelaxation, inhibition of platelet adhesion and aggregation and decreased neutrophil aggregation and adherence to the vascular endothelium.[41,54-56] NO is also a potent endogenous vasodilator

as it causes relaxation of vascular smooth muscle by increasing cellular cyclic guanosine monophosphate (cGMP) levels.[57] Results of a study by James *et al.*[58] suggest that the microvascular endothelium, which is in close proximity to the cardiac myocytes, may actually influence contractility through an NO-mediated mechanism.

The L-arginine ➡ NO pathway (figure 1) is believed to exert many of its physiological effects via stimulation of the enzyme soluble guanylyl cyclase (sGC).[59] NO and other NO donors activate sGC by binding to the heme moiety of this enzyme.[60] This results in increased cGMP concentrations that are associated with relaxation of vascular smooth muscle.[61] Also, NO donors increase cGMP in platelets and thereby inhibit their function.[62-64]

cGMP *per se* is believed to be cardioprotective via one or more of the following potential mechanisms:[65.66]

1. Inhibition of Ca^{2+} entry into the myocyte during reperfusion,
2. Prevention of muscle contracture that aids myocardial diastolic relaxation,
3. Optimization of myocardial energy substrate metabolism through inhibition of myocardial glycolysis, improved coupling between glycolysis and glucose oxidation, and more efficient use of glucose for ATP production.

Cardiac Ischemia-Reperfusion Injury

A 4-step mechanism is thought to play a role in IR injury:[67-73]

1. Impairment of vascular endothelium-dependent relaxation which causes edema formation due to the loss of barrier function,
2. Activation of oxygen free-radicals (OFRs) which promote neutrophil adhesion,
3. Enhancement of neutrophil/platelet aggregation and adhesion to the endothelium as this promotes injury of inflammation and microvascular permeability, which leads to end-organ dysfunction,
4. Myocardial stunning, which is a reversible regional or global post-ischemic mechanical dysfunction persisting for hours or days after reperfusion.

All these changes create an environment within the vessel that is predisposed to thrombus formation, increased vascular tone and ultimately coronary vasospasm or occlusion.[73]

Endothelial Function in IR Injury

Damage to the coronary vascular endothelium occurs from IR and is progressive with time.[74] This injury dramatically affects the basal as well as the stimulated generation of NO.[4-7] Pearson *et al.*[67] reported that NO causes vascular relaxation through a direct

action on vascular smooth muscle. Secondary to decreased vasorelaxation and impaired blood flow during reperfusion, baseline myocardial perfusion may not be fully restored in the post-ischemic region. This abnormality is referred to as the "no-reflow" phenomenon.[75]

Three mechanisms have been postulated to explain the decreased myocardial perfusion after IR injury:[1]

1. Interstitial and intracellular edema that may cause extravascular compression of the coronary arteries and arterioles,
2. Coronary smooth muscle damage that may increase vascular tone and impair relaxation,
3. Diminished NO release that may increase arterial tone and adversely affect coronary blood flow.

Free Radicals and Reperfusion Injury

Neutrophil activation and OFRs have been implicated as major contributors to IR injury.[6] Superoxide (O_2^-) and hydroxyl (OH^-) radicals, which are produced by myocytes, vascular endothelium and neutrophils, are released within minutes of the initiation of reperfusion. These radicals are highly reactive and cause lipid peroxidation, protein oxidation and DNA breakage.[76] The production of O_2^- and NO may both be stimulated during

reperfusion and these substances react to form $ONOO^-$ which can exacerbate oxidative injury (figure 1):[70]

$$NO + O_2^- \rightarrow ONOO^-$$

Peroxynitrite is a highly reactive, toxic and unstable compound which causes lipid peroxidation. It is also the most likely mediator implicated in oxidative injury.[70]

Role of Neutrophils in Myocardial Ischemia and Reperfusion

In the intact organism, ischemic myocardial injury initiates an acute inflammatory response in which neutrophils are major participants. Evidence indicates that the interplaying inflammatory reactions are augmented by reperfusion and that accumulating neutrophils contribute to myocardial damage. They do this by the release of ORFs, proteases and leukotrienes. In experimental models, interventions aimed at neutrophil inhibition or depletion can exert cardioprotective effects and some of these strategies raise hope for future clinical applications.[77-80]

Cardiotoxic Potential of Neutrophils

a) Neutrophils as a source of OFRs

Neutrophils contain an extensive cytotoxic armamentarium as their potential to destroy tissue is realized by concerted and synergistic effects of exocytosed granule constituents and generation of OFRs.[81-82] Highly reactive $OH^{\cdot}$ and O_2^{-} radicals are formed. Most stimuli that induce O_2^{-} generation by neutrophils also cause the release of myeloperoxidase (MPO) from the azurophil granules. This enzyme efficiently removes peroxide (H_2O_2) resulting in the production of hypochlorous acid. This acid is a powerful oxidant that may chlorinate or oxidize a variety of target molecules (e.g., primary amines/ ammonia). Chloramines (energetic oxidants) are produced by these mechanisms. Hypochlorous acid is considered to be primarily responsible for OFR-dependent cytotoxicity of neutrophils.[81-83] From this it follows that MPO activity can be used as a marker for neutrophil accumulation and neutrophil-mediated cytotoxicity in myocardial tissue. MPO activity can be evaluated according to a protocol set by Bradley *et al.*[35]

Neutrophil-derived OFRs can induce vascular contraction and in isolated perfused hearts they aggravate post-ischemic coronary dysfunction and left ventricular (LV) mechanical performance.[84-85] One of the mechanisms underlying the vasoconstricting properties of neutrophils may be the inactivation of endothelial NO by neutrophil-derived O_2^{-}.[86-87] OFRs are also believed to result in progressive inactivation of neutrophil-derived NO.[88]

b) Neutrophil-derived proteases

Although interest has focused on the cytotoxic potential of neutrophil-derived oxidants, neutrophil degranulation also releases several proteolytic enzymes into the extracellular milieu.[81-82] The serine proteinase elastase has been implicated most consistently in neutrophil-mediated tissue damage.[89] These proteinases contribute to tissue damage by direct alteration in target cell surface charge or by enhancing binding to cell membranes and extracellular matrix components. Elastase can hydrolyze a host of proteins in the extracellular matrix and may inhibit platelet function by proteolysis of platelet membrane glycoproteins.[90] Finally a synergism is thought to exist between neutrophil elastase and neutrophil-generated OFRs *in vivo*, since neutrophil-derived oxidants can inactivate the powerful anti-proteinases present in plasma and extracellular fluid.[91]

c) Arachidonic acid metabolites and PAF

In addition to OFRs and proteinases, activated neutrophils release several other pro-inflammatory mediators with a wide range of biological activities. Two of these, leukotriene B_4 and PAF are potent stimulants of neutrophil chemotaxis, adhesion to endothelial cells and oxidative metabolism.[92] They may serve to amplify neutrophil-mediated tissue injury and vascular permeability.[93] In addition, PAF produces aggregation and degranulation of platelets. Depressed coronary flow and LV function by PAF is likely to be dependent on these degranulation products.[94]

d) Myocardial neutrophil accumulation

In experimental models, neutrophil accumulation is accelerated by reperfusion.[95,96] In dogs the greatest rate of neutrophil localization is observed in the first hour of reperfusion. During reperfusion after sustained myocardial ischemia, neutrophil accumulation occurs preferentially in the subendocardial region and may correlate with infarct size.[97] Neutrophils are much larger and stiffer than erythrocytes as the cytoskeletal assembly after neutrophil activation is associated with additional decreases in cellular deformation. These hemorheological properties can promote physical trapping of neutrophils in myocardial capillaries after ischemia and reperfusion. Thus neutrophils may indeed contribute to the "no re-flow" phenomenon. Regional microvascular plugging by neutrophils is likely to be enhanced by other post-ischemic alterations including reduced endothelial NO production.[98]

Cardioprotection by Neutrophil Inhibition In Vivo

The cardioprotective potential of neutrophil inhibition in intact animals subjected to myocardial ischemia and reperfusion has been widely used to assess indirectly the role of neutrophils. There are many interventions that attenuate myocardial IR injury by their effects on neutrophils.[77]

a) Antioxidants

OFRs have been implicated in all manifestations of myocardial reperfusion injury. Although cardioprotective effects have been demonstrated in studies with antioxidant inhibitors of neutrophil oxidative metabolism, the precise role of these species in myocardial IR injury has not been clearly defined.[99-101]

b) Neutrophil depletion

Some reports, although not unchallenged, have indicated that neutrophil depletion can decrease reperfusion arrhythmias, improve post-ischemic perfusion and reduce the alteration in microvascular permeability after myocardial IR. Similar reports have also indicated that this depletion reduces myocardial infarct size when administered either before or at the time of reperfusion.[102] Caution is warranted however, as this protection may not be sustained. Therefore studies with long-term follow-up periods are warranted.[103]

c) Enhancement of endogenous NO availability

A topic by itself, the administration of NO donors or L-arginine has been shown to reduce tissue damage and neutrophil accumulation after experimental myocardial IR. One mechanism of this protection is the preservation of post-ischemic coronary

endothelial NO production of as this effect is likely to attenuate neutrophil-endothelial interactions.[104,105]

d) Adenosine

In addition to its known cardiovascular effects, adenosine is a strong inhibitor of neutrophil respiratory burst, which prevents neutrophil adherence and cytotoxicity to endothelial cells *in vitro*. Myocardial adenosine release increases in response to ischemia and thus may act as an endogenous modulator of inflammation. In agreement with this hypothesis, adenosine administration can reduce myocardial infarct size, post-ischemic neutrophil accumulation, coronary endothelial damage and contractile dysfunction.[106-109]

Clinical Applications

Despite the vast body of experimental evidence suggesting a role for neutrophils in myocardial IR, limited clinical data are available. The association between white blood cell count and the risk of myocardial ischemia was recognized two decades ago. With the advent of various revascularization techniques, the concept of myocardial IR injury has formed the background for a number of studies examining the role of neutrophils in patients with ischemic heart disease.[102]

Cardiopulmonary bypass (CPB) elicits complement activation, OFR generation and sequestration of neutrophils. Furthermore, CPB is associated with increased plasma

concentrations of neutrophil granule constituents (e.g., elastase) and priming of circulating neutrophils for increased OFR production. Activation of neutrophils and enhanced release of OFRs may therefore contribute to postoperative cardiac dysfunction, which still plays an important role in patients undergoing cardiac surgery.[110-113]

In summary, the acute phase response elicited *in vivo* by tissue injury is characterized by a pathophysiological cascade of inflammatory reactions such as cytokine production, fever, complement activation, leukocytosis and neutrophil infiltration. Neutrophils are among the cellular mediators implicated in myocardial IR injury, primarily since they may be an important source of OFRs. This hypothesis fits well with the other concepts of myocardial IR injury and is strengthened by observations that neutrophils accumulate in ischemic-reperfused myocardium and that inhibition of neutrophils can reduce myocardial damage. Further work is required to define the precise role of neutrophils in the pathophysiological processes occurring at various times during myocardial ischemia and reperfusion.[77]

Nitric Oxide and Ischemia-Reperfusion Injury

Regional Ischemic Heart Model

Based on the proposal that IR injury may result in decreased NO production, many attempts have been made to supplement NO levels during reperfusion in attempt to attenuate this injury. The direct infusion of NO (or L-arginine) has been shown to

significantly decrease both myocardial necrosis and neutrophil accumulation in a number of regional ischemia heart models.[2,7,13] Cardioprotection was speculated to occur secondary to inhibition of platelet and neutrophil accumulation, decreased neutrophil release of secretory products, direct cytoprotective effects of NO on both endothelial and myocardial cells and quenching of OFRs produced by neutrophils.[2] The beneficial effects of NO/L-arginine were also thought to be secondary to decreased oxygen demand by vasodilatation-induced hypotension and increased reflow through vasodilatation of the coronary circulation.

Global Ischemic Heart Model

The inclusion of an NO donor in blood cardioplegia (prior to hypothermic storage) improves ventricular performance and endothelial function, possibly via inhibition of neutrophil-mediated damage.[5] Endothelium-dependent relaxation of coronary arteries by acetylcholine is augmented by L-arginine when infused during the early reperfusion period. In the global ischemia model, NO is thought to counteract cellular damage by blocking the formation of OFRs in addition to their neutralization. This decreases lipid peroxidation and retards the damaging effects of IR injury.[8,10]

Blood Reperfusion Model

There is limited evidence available regarding the cardioprotective effects of NO/cGMP augmentation in blood reperfused hearts subjected to cardioplegic arrest and prolonged

hypothermic storage.[116-122] NO increases survival of hearts (stored for 24 hrs) when perfused in the presence of nitroglycerin (NTG) and a cAMP analogue. Oz *et al.*[116] used an orthotopic baboon cardiac transplantation model where 0% (n=4) of untreated hearts and 80% (n=5) of treated hearts (NTG and cAMP) survived the perioperative period. Survival of hearts in this study was defined by the ability to successfully wean the animals from cardiopulmonary bypass. Unfortunately, the outcome measurements used in the study and the lack of follow-up work weaken the evidence for NO-mediated protection with blood reperfusion in this model.

Two groups have investigated the effects of NO/cGMP supplementation on IR injury in a heterotopic rat heart transplantation model.[118-119] Szabo *et al.*[118] reported that after 1 hr of ischemic preservation, an NO donor improved recovery of myocardial and endothelial function during early reperfusion after heart transplantation. They also concluded that initial treatment with L-arginine had a persisting beneficial effect against reperfusion-induced graft coronary endothelial dysfunction. Pinsky *et al.*[119] examined survival (not mechanical function) of blood reperfused hearts stored for 12 hrs. The addition of NTG (NO donor) to a balanced salt preservation solution enhanced graft survival in a time and dose-dependent manner, with 92% of hearts supplemented with NTG surviving 12 hrs of preservation versus only 17% in its absence. There are no studies looking at the effects of NO on mechanical function in hearts subjected to long-term storage and reperfusion with blood.

NOS Inhibition

The effects of NOS inhibitors (e.g., L-nitro-arginine methyl ester or "L-NAME") on cardiac IR injury have been investigated in most models of IR injury.[114-115] The controversy on NO-mediated cardioprotection arises with the finding that NOS inhibition under certain circumstances can actually decrease infarct size. The mechanism of L-NAME is considered to involve the release of adenosine, which confers cardioprotection in accordance with an ischemic "preconditioning" effect.[114] Schulz and Wambolt[15] reported that the recovery of post-ischemic LV function could be improved by reducing NO synthesis in a rabbit heart model. They suggested that inhibition of NO synthesis (with L-NAME) could offer prolonged protection, whereas stimulation of NO production (via L-arginine) provided only brief protection. The pharmacologic effectiveness of these divergent strategies may be related to the prevention of $ONOO^-$ generation during reperfusion (figure 1).[15] Interestingly, there is equally compelling evidence implying that endogenously produced NO (by NOS) participates in the modulation of myocardial IR pathophysiology, leading to cardiac necrosis. Therefore, a paradox exists with respect to NO-mediated cardioprotection with the discrepancy between studies showing cardioprotection by NOS inhibitors (compared with those demonstrating harmful effects) probably relating to experimental models. This emphasizes the need for further study in this area.

In the blood reperfusion situation the effects of infusions of L-arginine and L-NAME in isolated blood-reperfused neonatal lamb hearts following 2 hrs of cold cardioplegic

ischemia have been investigated. L-arginine-treated hearts showed significantly better recovery of LV systolic, diastolic and endothelial function when compared with untreated hearts. The addition of L-NAME was found to negate the beneficial effects of L-arginine in the blood reperfusion situation.[117]

In summary, it remains unclear whether exogenously supplied NO is beneficial in myocardial reperfusion. When NO released from endothelial cells is depressed (as a result of synthesis inhibition), increased tissue damage is noted in cardiac IR injury. However, we know that NO functions as an injurious agent during reperfusion through the production of $ONOO^-$.[114] Much research is currently being done in this specific area in to investigate the apparent contradiction.

Objectives

Cardioprotection after Cardioplegic Arrest and Prolonged Hypothermic Storage in the Presence of Crystalloid Reperfusion

Work by Ali *et al*[66] suggests that the key to extending the safe ischemic time of cardiac allografts is the elucidation of the mechanisms underlying the etiology of myocardial IR injury. They determined that activation of the NO/cGMP pathway by sodium nitroprusside (SNP) protects hearts subjected to cardioplegic arrest and prolonged hypothermic storage. Apart from reperfusion in the presence of crystalloid, all experiments were completed in a scenario analogous to clinical donor-heart preservation.

There is strong evidence that the protective effect of SNP arises as a consequence of activation of the NO/cGMP pathway. The precise mechanism by which activation of this pathway leads to cardioprotection is unknown. Data support a direct myocardial action of SNP. Elevation of cGMP content has been shown to influence favorably myocardial energy substrate metabolism by reducing the rate of myocardial glycolysis.[66] This should in turn improve coupling between glycolysis and glucose oxidation and attenuate proton production and acidosis.[125] The attenuation of proton production then improves recovery of LV work and cardiac efficiency of the post-ischemic heart.[126]

The study by Ali *et al*[66] supports the protective effect of SNP during storage, non-working reperfusion and working reperfusion of transplanted hearts. Experiments were performed in isolated, fatty acid perfused working rat hearts in the absence of bloodborne cellular elements. This effectively eliminated any major contribution of neutrophils to IR injury. The initial objective of the current study will be to confirm the cardioprotective effects (after prolonged storage) of SNP in the presence of crystalloid reperfusion.

Cardioprotection after Cardioplegic Arrest and Prolonged Hypothermic Storage in the Presence of Blood Reperfusion

Most studies on myocardial preservation and IR for transplantation have been performed with crystalloid-perfused isolated heart preparations.[53] The major advantage for these preparations are the well controlled conditions for assessing experimental variables. However, the crystalloid model differs from the clinical transplant reperfusion situation.

The exclusion of platelets and neutrophils eliminates major influencing factors of myocardial ischemia and reperfusion.[127] A heterotopically transplanted rat heart model can be used to simulate the clinical situation where the heart is transplanted and receives whole blood pulsatile reperfusion.[53] It also allows the design of studies to address specifically the effects of neutrophils and other bloodborne cellular elements in cardiac IR injury. The original heterotopic transplant model was developed by Ono-Lindsey in 1969.[128] It is the aim of the current study to investigate the cardioprotective effects of NO/cGMP activation in the presence of bloodborne cellular elements in a heterotopic rat heart transplant model.

Clinical Application

Heart transplantation is an excellent therapeutic modality for select patients with end-stage heart disease. The current acceptable ischemia times for liver (12 hrs) and renal (24-48 hrs) allografts are significantly longer than that for cardiac allografts (4-6 hrs). Studies of myocardial IR injury and the development of novel pharmacologic strategies to prolong the maximum "safe" ischemia time for cardiac allografts have many potential clinical benefits. Wasting of a limited number of available organs as well as overcoming barriers to long distance organ procurement and sharing would be avoided. Furthermore, improved myocardial protection during hypothermic storage and reperfusion would decrease the incidence of early graft failure resulting in an improved recipient morbidity and mortality after transplantation.

Materials and Methods

Animals

Male Lewis rats (n=95), 7-8 weeks old, and weighing 250-350 g were used. All animals were fed *ad libitum* and housed at room temperature under a 12 hr light dark cycle. The animals received care according to the Canadian Council on Animal Care and all procedures were approved by the University of Alberta Health Sciences Animal Policy and Welfare Committee.

Preparation of Donor Heart

Hearts were excised after the induction of anesthesia with intraperitoneal pentobarbital (240 mg). After laparotomy and bilateral thoracotomy, the rats were bled by cutting the abdominal aorta and the thoracic organs were subsequently removed. The aortae were cannulated and retrograde perfusion using a Krebs-Henseleit solution (11 mM glucose and 2.5 mM Ca^{2+}) was initiated at a constant perfusion pressure of 60 mm Hg. The Krebs solution was kept at 37^{0}C, pH 7.4 and gassed with a 95% oxygen 5% carbon dioxide mixture. Next, the pulmonary artery was transected and all cardiac veins tied and divided. Both lungs were removed after hilar ligation.

Heterotopic Transplantation

Recipient rats were premedicated with 0.05 mg•kg^{-1} of atropine intramuscularly, and then underwent induction of anesthesia with inhalation of halothane in oxygen. Using clean microvascular technique, a heterotopic heart transplantation was carried out. After the abdominal cavity was opened, the abdominal aorta and inferior vena cava were mobilized. The graft aorta and pulmonary artery were anastomosed separately end-to-side to the recipient aorta and vena cava with a continuous 10-0 nylon suture. After unclamping, initial ventricular fibrillation spontaneously converted to normal sinus rhythm in 95% of recipients. The animals received 5 mL of Ringer's Lactate through the dorsal vein of the penis for volume maintenance throughout the operation. Body temperature was supported with a heating blanket and warmed fluid infusion.

Postoperative Care of Animals

Following transplantation, the animals recovered in a warm environment for 60 min. Expected immediate mortality was 5% due to hypovolemic shock or potential vascular thrombosis. All hearts were reperfused with blood for 60 min. Each heterotopic organ hearts was then excised after the induction of anesthesia with another intraperitoneal dose of pentobarbital (240 mg). The recipient rats were bled at this point by cutting the abdominal aorta and inferior vena cava. The aortae (transplanted hearts) were then cannulated and retrograde perfusion using Krebs-Henseleit solution was initiated as described above for preparation of the donor heart.

Perfusate Solutions

Perfusion of the hearts during Langendorff mode was performed using a Krebs-Henseleit solution containing NaCl (118 mM), KCl (4.7 mM), KH_2PO_4 (1.2 mM), $MgSO_4$ (1.2 mM), $CaCl_2$ (2.5 mM), $NaHCO_3$ (25 mM) and glucose (11 mM). Hearts were arrested using ice-cold St. Thomas II cardioplegic solution. The working mode perfusate was a Krebs-Henseleit solution with the addition of palmitate (1.2 mM) pre-bound to bovine serum albumin. Insulin (100 $\mu U \cdot mL^{-1}$) was added to all working mode solutions.

Perfusion Protocols

All hearts were perfused on an isolated heart perfusion apparatus (figure 2). There were 4 groups of hearts subjected to specific perfusion protocols. The protocols differed between hearts and contained various combinations of the following perfusion phases (refer to figure 3):

- Initial Langendorff perfusion (post donor cardiectomy) that comprised of a 10 min period of stabilization in the non-working Langendorff mode. **(L_I PHASE)**
- Storage phase in which hearts were arrested with 25 mL of ice cold St. Thomas II cardioplegic solution (110 mM NaCl, 10 mM $NaHCO_3$, 16 mM KCL, 16 mM $MgSO_4$, 1.2 mM $CaCl_2$, pH 7.8). This was administered at a constant perfusion pressure of 60 mm Hg and thereafter the hearts were

removed from the perfusion rig and stored in cold St. Thomas II solution at 4^0C for 5-8 hrs. Hearts reperfused with crystalloid (CR hearts) were stored for 8 hrs, whereas hearts reperfused with blood (BLR hearts) were stored for 5 hrs. **(S PHASE)**

- Total ischemic time was extended by 1.5 hrs (BLR hearts) during the heterotopic transplantation stage. The heart was maintained cold and arrested (10-14 ^{0}C) by wrapping it in cold gauze which was regularly irrigated with cold saline (4^0C). **(HT PHASE)**
- Blood reperfusion of the heterotopically transplanted hearts was maintained for 60 min after cross-clamp removal. The hearts were in nonworking, nonrecirculating mode and perfused with the recipient's blood at 37^0C. **(BLR PHASE)**
- Repeat Langendorff perfusion that comprised of a 10 min period of stabilization in the non-working Langendorff mode. **(L_2 PHASE)**
- Working mode reperfusion for 60 min in which hearts were paced at a rate of 300 beats•min^{-1} (Grass SD9 stimulator). Reperfusion in the working mode was performed at a constant aortic afterload (80 mm Hg) and preload (11.5 mm Hg). **(W PHASE)**

During the Langendorff (10 min) and working mode reperfusion, hearts were perfused at 37^0C under aerobic conditions. A compliance chamber was attached to the aortic outflow line and filled with air in order to maintain LV developed pressure at approximately 60 mm Hg. Perfusate was delivered to the oxygenator with a peristaltic pump

(Masterflex, Cole-Palmer Instrument Company). The peristaltic pump was supplied by a reservoir collecting coronary and aortic flows as well as overflow from the oxygenator (refer to figure 2). At the end of each perfusion protocol, the hearts were frozen with Wollenberger clamps cooled to the temperature of -179 ^{0}C . They were then ground into a fine powder and stored at -80 ^{0}C.

Experimental Groups (Crystalloid Reperfusion)

A. Fresh Hearts (figure 3)

Eight freshly excised hearts that were not subjected to cardioplegic arrest, storage or transplantation were perfused in the **(L_1)** mode for 10 min and then in the working mode **(W)** for 60 min to assess coronary flow and mechanical function under normal aerobic conditions.

B. Stored Crystalloid Reperfused Hearts (CR hearts, figure 3)

Hearts were subjected to cardioplegic arrest, hypothermic storage (8 hrs) and Langendorff reperfusion on the rig **(L_1 + S + L_2 + W).** All hearts were randomly assigned to CR-treated (n=6) and CR-untreated (n=6) groups. The treated hearts were exposed to sodium nitroprusside (SNP 200 μM) throughout all phases of the perfusion protocol.

Experimental Groups (Blood Reperfusion)

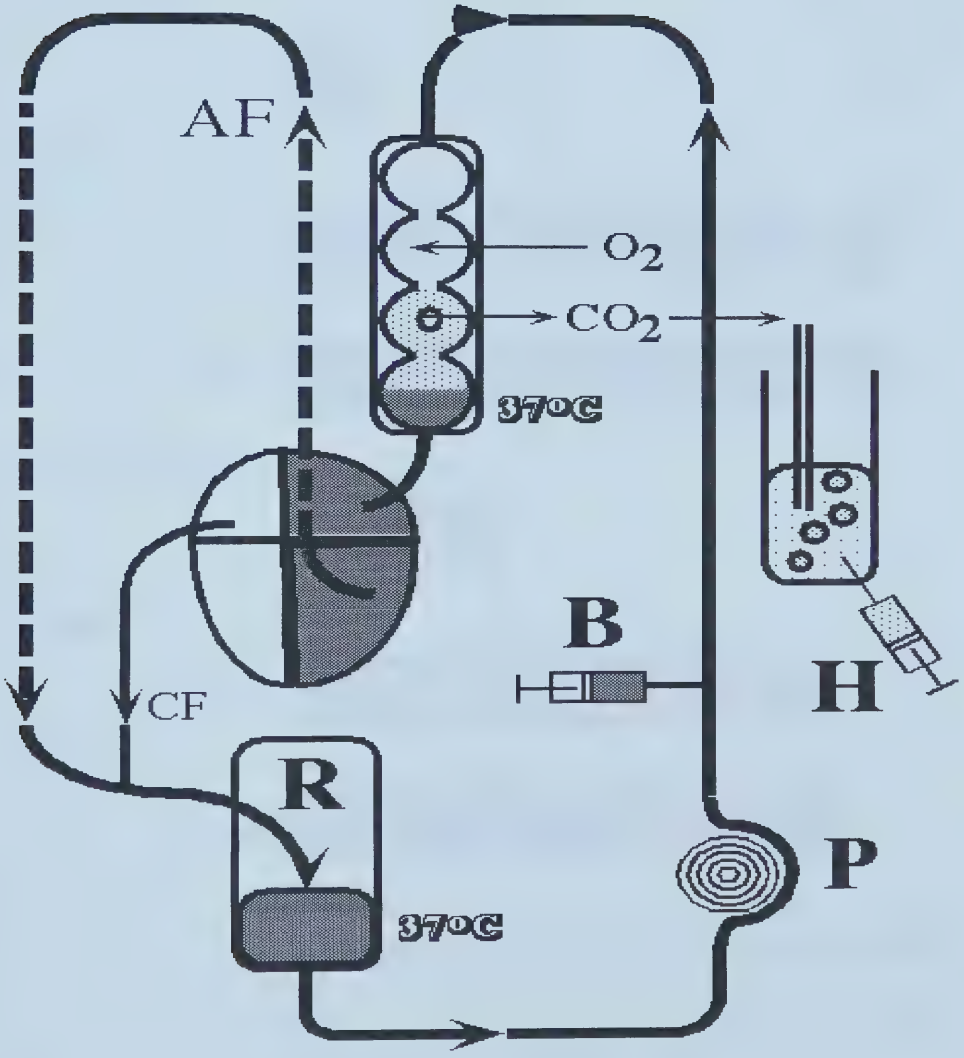
C. Fresh Transplants (figure 3)

Hearts were subjected to cardioplegic arrest, heterotopic transplantation and blood reperfusion prior to Langendorff and working mode reperfusion on the rig **(L_1 + HT + BLR + L_2 + W).** There was no storage period. Mechanical and metabolic function were assessed *ex vivo* on the heart perfusion apparatus. The objective was to assess the effects of a 1 hr transplantation procedure on mechanical function in Fresh Hearts.

D. Stored Transplanted Hearts (BLR hearts, figure 3)

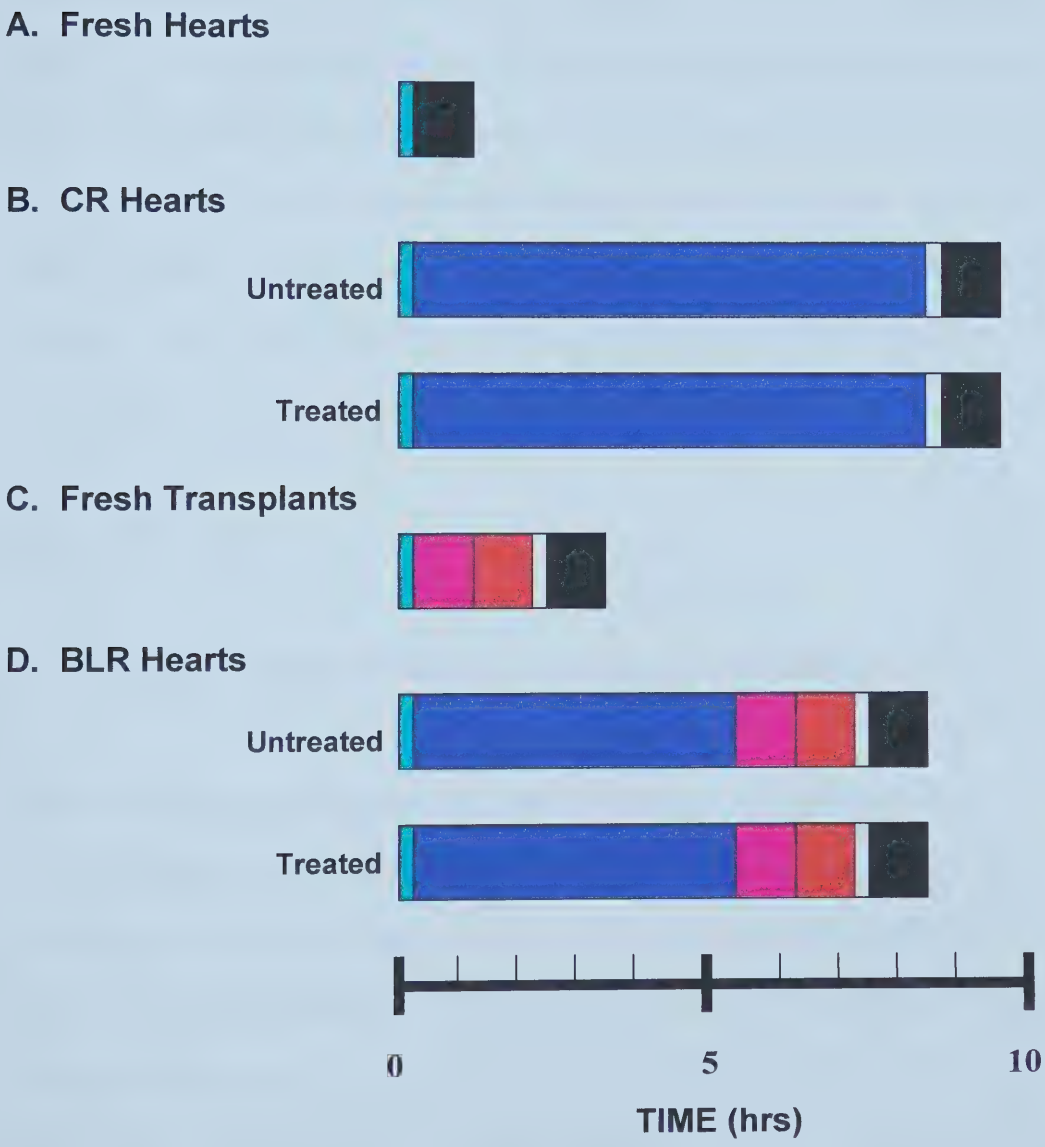
Hearts were subjected to cardioplegic arrest, hypothermic storage, heterotopic transplantation and blood reperfusion prior to Langendorff and working mode reperfusion on the rig **(L_1 + S + HT + BLR + L_2 + W).** Prior to arrest, hearts were assigned to BLR-treated (n=6) and BLR-untreated (n=5) groups. Treated hearts were exposed to SNP (200 μM) during initial stabilization, arrest and storage. Just prior to blood reperfusion a terminal "hot shot" (37^0C) of SNP (20 mL at 200 μM) was given via direct cannulation of the aorta. The coronaries were perfused at a pressure of 60 mm Hg for 2 min. Zaprinast (1 mg•kg^{-1}) was administered to the recipient animals (BLR-treated group) by injection of the inferior vena cava 5 min prior to blood reperfusion.

Figure 2. Perfusion Apparatus



P = Pump; R = Reservoir for Perfusate; AF = Afterload Line; H = Hyamine; CF = Coronary Flow; O = Oxygenator

Figure 3. Diagram Representing Perfusion Protocols of the Various Experimental Groups



Green = initial Langendorff Perfusion (L_1); Blue = Hypothermic Storage Phase (**S**); Pink = Heterotopic Transplantation Phase (**HT**); Red = Blood Reperfusion Phase (**BLR**); White = Repeat Langendorff Perfusion (L_2); Black = Working Mode Perfusion Phase (**W**)

Mechanical Function

Mechanical function was assessed in all experimental groups on the isolated heart "rig" (figure 2). Cardiac output and aortic and flows were measured using flow probes within the preload and afterload lines (Transonic Systems Inc.). Coronary flows were measured indirectly as the difference between cardiac output and aortic flows. Aortic systolic and diastolic pressures were recorded using a pressure transducer (Gould P21). Left ventricular minute work ($L \cdot min^{-1} \cdot mm\ Hg$) was calculated as cardiac output X LV developed pressure.

Glucose Metabolism

A. Measurement of Myocardial Glycolysis and Glucose Oxidation (figure 4)

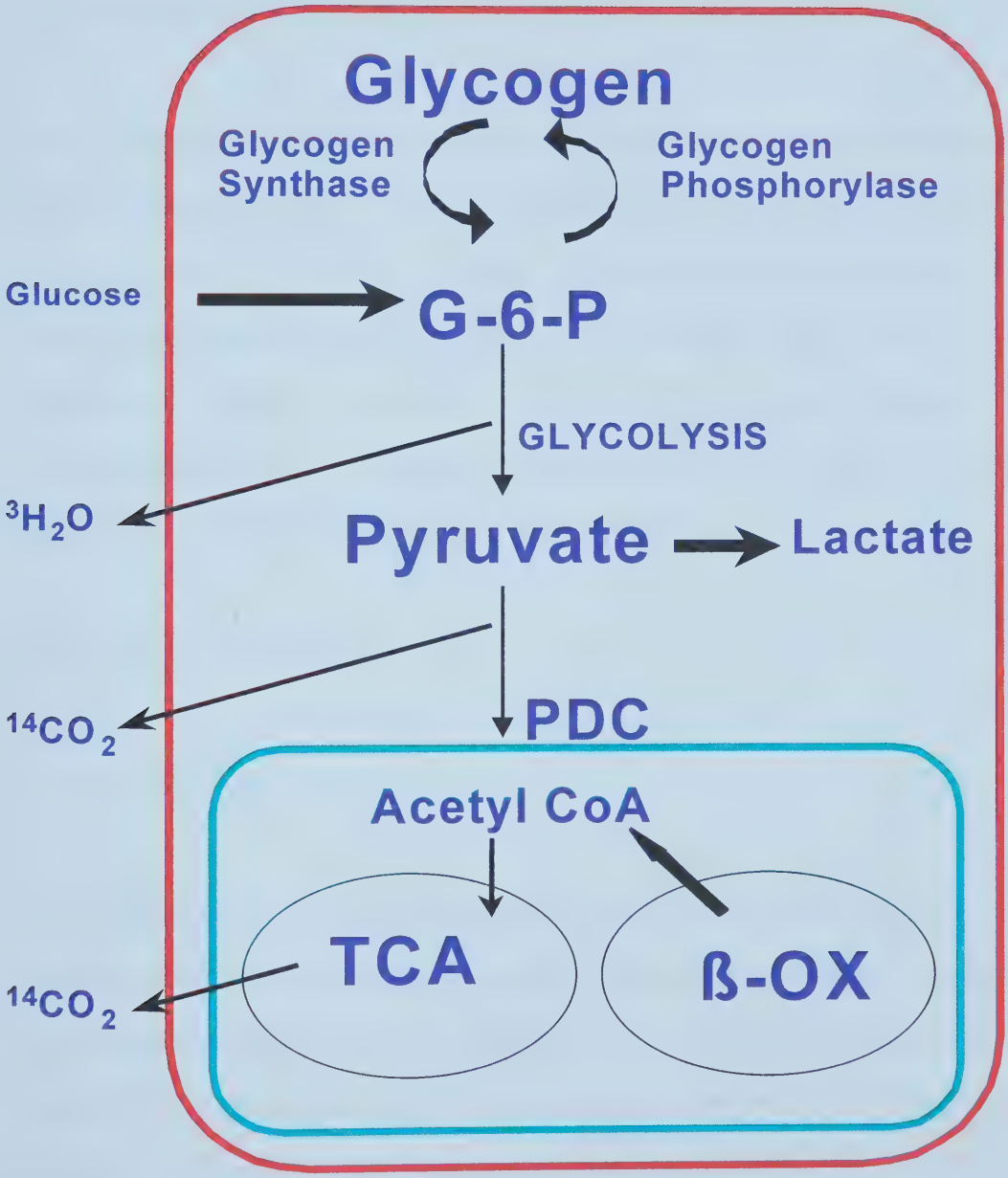
Glycolysis and glucose oxidation were measured in hearts perfused with solution containing trace amounts of [^{3}H]glucose and [^{14}C]glucose.[125] Glycolysis and glucose oxidation were measured simultaneously by quantitative collection of $^{3}H_2O$ (produced at the enolase step of glycolysis) and $^{14}CO_2$ (produced at the pyruvate dehydrogenase step of glucose oxidation and in the TCA cycle, figure 4). Samples of perfusate (3 mL) were removed at 10 min intervals directly from the perfusion rig without exposure to air. They were subsequently injected into glass vials below mineral oil (3 mL) in order to prevent the escape of gaseous CO_2. The CO_2 escaping into the gaseous phase and exiting into the oxygenation chamber was collected by passing the gas through a 1 M hyamine hydroxide

trap (40 mL). Samples were collected from the trap for $^{14}CO_2$ determination at 10 min intervals.

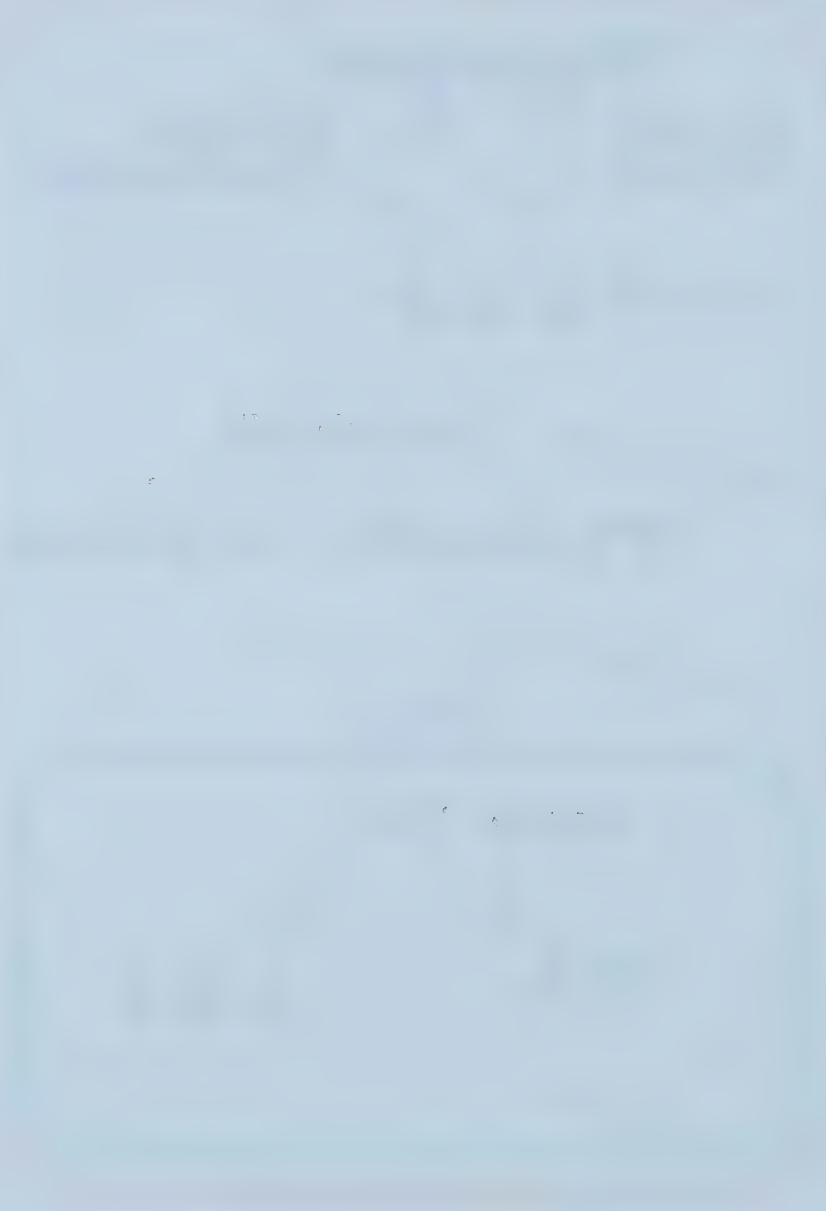
Average rates of glycolysis and glucose oxidation were calculated from $^{3}H_2O$ and $^{14}CO_2$ accumulation between 10 and 60 min of aerobic, working mode perfusion. In order to measure glycolysis, $^{3}H_2O$ in perfusate samples (0.1 mL) was separated from [^{3}H]glucose using columns containing Dowex 1-4X anion exchange resin. Effluent from the columns was collected into a scintillation fluid (5 mL) and counted for 5 min in a Beckman LS 6500 scintillation counter. Average rates for glycolysis were expressed as μmol glucose metabolised•min^{-1}•g dry weight^{-1}.

Glucose oxidation was measured by taking perfusate samples (1 mL) and injecting them into 9 N H_2SO_4 (1 mL) contained in sealed 25 mL flasks with a center well of 1 M hyamine hydroxide (400 μL). The purpose of the hyamine was to trap the $^{14}CO_2$ released from bicarbonate in the samples. After gentle shaking (1 hr), the hyamine trap was again placed in scintillation fluid (5 mL) and counted for 5 min in a Beckman LS 6500 counter. Steady state rates for glucose oxidation were also expressed as μmol glucose metabolised•min^{-1}•g dry weight^{-1}.

Figure 4. Metabolic Pathways



G-6-P = Glucose-6-Phosphate; PDC = Pyruvate Dehydrogenase Complex; TCA = Tricarboxylic Acid Cycle; *B*-OX = *Beta* Oxidation



B. Calculation of Proton Production

Ideally, every molecule of glucose metabolised by glycolysis would be coupled 1:1 with glucose oxidation. This results in a most efficient use of glucose for ATP production with a net production of no protons. However, if these two pathways are uncoupled (glycolysis exceeding glucose oxidation) there is a net production of two protons for every molecule of glucose passing through glycolysis without subsequent oxidation.[129] Ultimately, the rate of proton production by glycolytically-derived ATP hydrolysis can be calculated as 2 X (rate of glycolysis - rate of glucose oxidation).

Nucleotide and Protein Assays

A. Myocardial cGMP Content

Frozen (200 mg), pulverised heart tissue was kept cold in liquid nitrogen. The vials containing the heart tissue were then warmed up to ice temperature. The tissue was then homogenised in a Hepes-perchloric acid solution (pH = 7.4, 2.2% PCA) in the presence of 5 mM EDTA. The samples were allowed to stand for 15 min and then centrifuged at 10,000g for 2 min at 4 ^{0}C. A 250 µL aliquot of sample was neutralised (pH 7.0-8.0) with a 1.1 M K_3PO_4 and centrifuged at 10,000g for 2 min at 4 ^{0}C. A 50 µL aliquot of the supernatant was used for the cGMP immunoassay (cGMP immunoassay kit from Cayman Chemical).

The enzyme immunoassay is based on the competition between free cGMP and a cGMP tracer for a limited number of cGMP-specific rabbit antiserum binding sites. The amount of tracer is kept constant as the free cGMP in the sample varies. Essentially, sample and tracer are added to a plate (96 wells) containing antiserum binding sites for cGMP. The plate is then washed to remove any unbound reagents and then Ellman's reagent (substrate to acetylcholinesterase) is added to each well. The product of this reaction produces a yellow colour and absorbs at 412 nm. Absorbance (determined spectrophotometrically) is inversely proportional to the amount of free cGMP in each well. The absorbance is then compared to a standard curve of cGMP in order to determine cGMP contents. The content of cGMP in the myocardial tissue is expressed as pmol • mg protein^{-1}.

Tissue protein content was determined by BCA protein assay on the remaining pellet from the cGMP perchloric acid extraction protocol described above. The frozen pellet was dissolved by boiling in 500 μL 2 N NaOH for 10 min. This solution was then cooled to room temperature and neutralised (pH 6.8-7.0) with 2 N HCL. The samples were centrifuged at 10,000g for 2 min at 4 ^{0}C and the supernatants assayed with BCA reagent for protein content against a standard curve.[130]

B. Myeloperoxidase Assay

Myeloperoxidase (MPO) activity can be used as a marker for neutrophil infiltration

into tissues. MPO activity was evaluated as described by Bradley *et al.*[35] Frozen (100 mg), pulverised heart tissue was kept cold in liquid nitrogen. This tissue was homogenised in 1.5 mL of 50 mM potassium phosphate buffer (pH=6.0). The homogenate was centrifuged at 10,000g for 10 min and the pellet was suspended in 1 mL of the same potassium phosphate buffer containing 0.5% hexadecyltrimethylammonium bromide (HDTA, Sigma). The HDTA negates the pseudoperoxidase activity of hemoglobin and solubilizes membrane-bound MPO. The suspensions were treated with 3 cycles of freeze-thawing, sonicated on ice for 10 sec, and centrifuged at 12,000g for 10 min. MPO activity was determined in the supernatants.[131]

Briefly, 0.1 mL of supernatant was mixed with 2.9 mL of potassium phosphate buffer (50 mM, pH=6.0) containing 0.19 $mg \bullet mL^{-1}$ of o-dianisidine chloride and 0.0005% H_2O_2 (hydrogen peroxide) as a substrate for MPO. Oxidised o-dianisidine forms a stable chromophore absorbing at 460 nm wavelength. The absorbance was determined over 2 min with values of tissue MPO activity obtained by comparison with standard concentrations of o-dianisidine in the presence of excess H_2O_2. One unit of MPO activity was defined as that required to degrade 1 μmol of H_2O_2 per min at 25^0C.[5] Tissue protein content was determined by BCA protein assay.[3] Results were expressed as $mU \bullet mg\ protein^{-1}$.

Sources of Drugs and Reagents

Sodium nitroprusside (SNP) was prepared by adding precalculated amounts of SNP · $2H_2O$ (Sigma Chemical Company) to either the Krebs-Henseleit solution or the St. Thomas' cardioplegia to obtain final concentrations of 200 μM as required by the experimental protocol. Myocardial cGMP content was determined by immunoassay with commercially available kits from Cayman Chemical. D-[5-^{3}H]glucose and D-[U-^{14}C]glucose were purchased from Du Pont-New England Nuclear (Boston, MA). Bovine serum albumin was purchased from Boehringer Mannheim. Hyamine hydroxide and Ecolite counting scintillant were obtained from Biomedicals Canada Ltd (Mississauga, Ontario). All other chemicals used in perfusion protocols and enzyme/nucleotide assays were purchased directly from Sigma Chemical Company.

Statistical Analysis

Data are expressed as the mean ± the standard error of the mean. Comparisons between pairs of groups were performed using the Student's t-test. Significant differences in LV work between the treatment groups (at the individual time points during the 60 min perfusion period) were estimated by analysis of variance. If significant, selected data sets were compared by Bonferroni's Multiple Comparisons test followed by Bartlett's test that the variances were homogenous. Differences were considered significant if p values < 0.05.

Results

Crystalloid Reperfusion

Recovery of Left Ventricular Function

All hearts in the CR-treated group spontaneously converted to a sinus rhythm after 2-3 min of reperfusion (Langendorff mode, $\mathbf{L_2}$) on the heart perfusion apparatus. The majority of hearts in these groups returned to a normal rate of 300 beats•min^{-1}. In the rare instance that reperfused hearts in these two groups did not return to a normal rate, they were paced at 300 beats•min^{-1} with a Grass SD9 stimulator. Of the CR-untreated group, 33% of hearts did not spontaneously convert to a normal sinus rhythm and remained in intractable ventricular fibrillation. These hearts were unable to be paced and demonstrated an LV work index of essentially 0.00 L • min^{-1} • mm Hg.

Baseline LV function in rat hearts (Lewis strain) was not significantly different from that for the more commonly used Sprague-Dawley strain. Optimal function for all CR hearts was determined in the Fresh Heart group (figure 5). The LV work index peaked in all hearts at 50 min of working mode reperfusion, which in the Fresh Heart group was 7.37 L • min^{-1} • mm Hg. CR-untreated hearts recovered to only 33% of the optimum work level measured in the Fresh Heart group (unstored). Cardiac output (mL•min^{-1}) and aortic flow (mL•min^{-1}) were also significantly reduced in the untreated group of hearts

(table 1). Recovery of left ventricular function in CR-treated hearts was improved by SNP (200 μM) when present throughout all phases of the perfusion protocol. Hearts in the CR-treated group recovered to 70% of optimal function, which was a statistically significant improvement when compared with the CR-untreated group ($p<0.05$). There was also a trend towards improved cardiac output, aortic flow and coronary vascular conductance in the SNP treated group (table 1).

Glucose Metabolism

Metabolic data for Fresh Hearts was taken from Finegan *et al.*[125] Rates for glycolysis and glucose oxidation in Fresh Hearts is reported as 4.0 and 0.50 μmol•g dry weight^{-1}•min^{-1} respectively. There was a trend towards decreased rates of glycolysis in the CR-treated group compared with the Fresh Heart and CR-untreated groups (figure 6). Glucose oxidation rates were significantly depressed in both CR-untreated and treated groups of hearts compared with the Fresh Heart group, although this suppression of glucose oxidation was not nearly as marked in the CR-treated group. Also noted was a trend towards decreased rates of proton production in CR-treated hearts to levels comparable with the Fresh Heart group.

Figure 5. Effect of Sodium Nitroprusside on the Recovery of LV Work in Fresh Hearts and in Hearts Subjected to Cardioplegic Arrest, Prolonged Hypothermic Storage and Reperfusion with Crystalloid

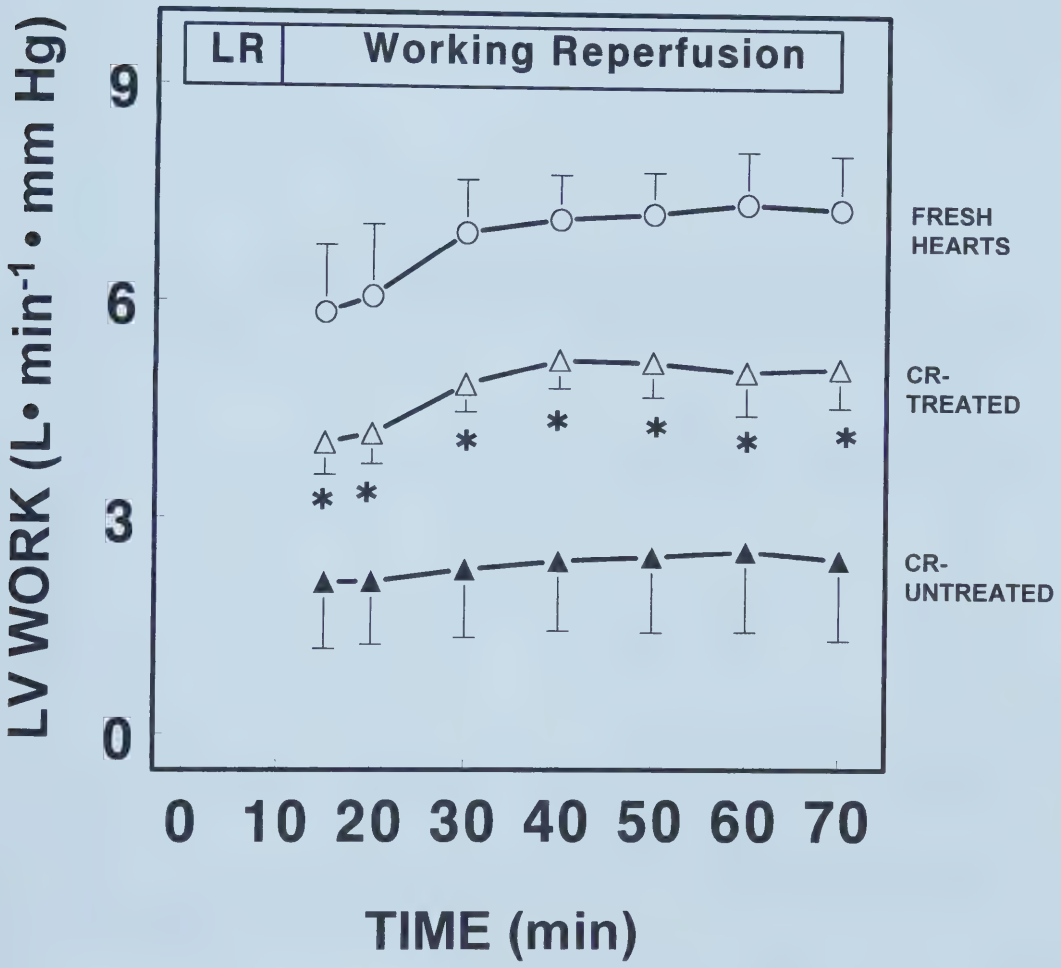


Figure 5. Left ventricular function in working mode reperfusion (**W** phase). Data are shown for Fresh Hearts (non-stored, open circles, n=4), CR-untreated hearts (8 hr storage, crystalloid reperfused, black triangles, n=6) and CR-treated hearts (8 hr storage, crystalloid reperfused, open triangles n=6). **LR** represents the $\mathbf{L_1}$ phase for Fresh Hearts and the $\mathbf{L_2}$ phase for CR-untreated and treated hearts. Data are shown as the mean ± the standard error of the mean. ✱ There was a statistically significant improvement in the recovery of mechanical function in CR-treated hearts compared to CR-untreated hearts ($p < 0.05$).

Table 1. Effect of Sodium Nitroprusside on Recovery of Myocardial Function in Fresh Hearts and in Hearts Subjected to Cardioplegic Arrest, Prolonged Hypothermic Storage (8 hr) and Reperfusion with Crystalloid

Group	n	CO ($mL \cdot min^{-1}$)	AF ($mL \cdot min^{-1}$)	CF ($mL \cdot min^{-1}$)	CVC ($mL \cdot min^{-1} \cdot mmHg^{-1}$)
Fresh	4	66.3 ± 4.2	46.0 ± 4.2	20.3 ± 2.8	0.23 ± 0.03
CR-untreated	6	23.8 ± 9.9	8.30 ± 5.0	15.5 ± 5.1	0.22 ± 0.05
CR-treated	6	42.5 ± 5.1*	17.7 ± 3.6	24.8 ± 2.6	0.29 ± 0.04

Data are shown as the mean ± the standard error of the mean of the given number of observations (n) at the end of 60 min of working reperfusion. * Significance: $p < 0.05$ by analysis of variance versus the untreated group.

CO = cardiac output; AF = aortic flow; CF = coronary flow; CVC = coronary vascular conductance; SNP = sodium nitroprusside (200μM)

Figure 6. Effect of Sodium Nitroprusside on Myocardial Glucose Metabolism and Proton Production in Fresh Hearts and in Hearts Subjected to Cardioplegic Arrest, Prolonged Hypothermic Storage and Reperfusion with Crystalloid

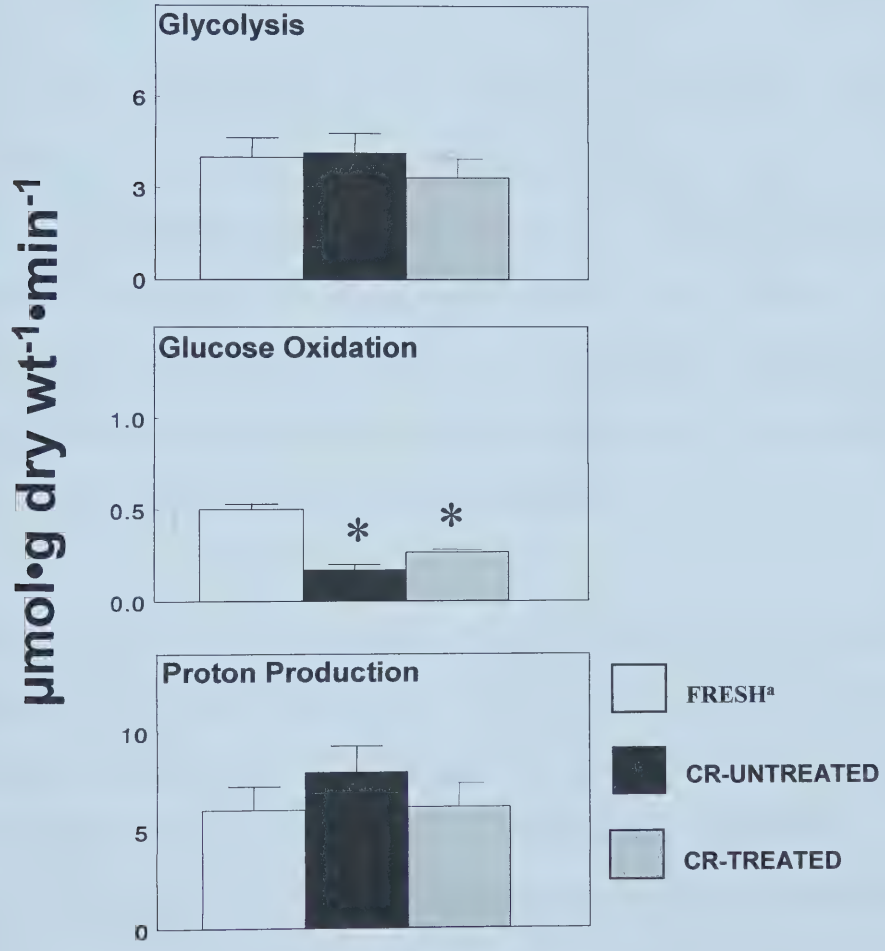


Figure 6. Myocardial glucose metabolism (µmol•g dry wt^{-1}•min^{-1}) in Fresh Hearts and crystalloid reperfused rat hearts. All results were measured and averaged over 60 min of working mode reperfusion. Data are shown for Fresh Hearts (non-stored), CR-untreated hearts (8 hr storage, n=6) and CR-treated hearts (8 hr storage, n=6). Data are shown as the mean ± the standard error of the mean. There were no significant differences in glycolysis, glucose oxidation and proton production between treated and untreated groups. * Rates of glucose oxidation were significantly depressed in both CR-untreated and treated groups compared to the Fresh Heart group ($p<.05$). [a] Data for Fresh Heart group (Sprague Dawley strain) taken from Finegan *et al.*[125]

Blood Reperfusion

Recovery of Left Ventricular Function

All hearts in the Fresh Transplant and BLR-untreated and treated groups spontaneously converted to a normal sinus rhythm after cross-clamp release and 2-3 min of blood reperfusion in the abdomen of the recipient rat. All hearts in these groups returned to a normal rate of 300 beats•min^{-1}. Overall operative mortality was 15% with no significant differences between the groups. Cause for mortality was bleeding (10%) and graft thrombosis (5%). Hearts suffering these surgical complications were not included in groups for subsequent functional and metabolic analysis.

A storage period of 8 hrs resulted in hearts that were unable to function ("stone hearts") during working mode reperfusion. The originally planned storage period for BLR hearts was subsequently decreased to 5 hrs which produced a level of damage from which hearts could be expected to recover function after blood reperfusion. This period of hypothermic storage remained consistent between BLR-untreated and treated groups.

Optimal function for all BLR hearts was determined in the Fresh Transplant group (figure 7). LV work peaked in the Fresh Transplant group at 30 min of working mode reperfusion (6.51 L • min^{-1} • mm Hg). Whereas SNP was found to be cardioprotective in crystalloid reperfused hearts, it did not enhance the recovery of BLR-treated hearts (5 hr storage). LV work in the BLR-untreated group recovered to 25% of the Fresh Transplant

group. Cardiac output and aortic flows were severely depressed in this group of hearts (table 2). Hearts in the BLR-treated group (SNP 200 μM + zaprinast 1mg•kg^{-1}) also demonstrated a very poor recovery of LV function (22%) which was not significantly different from the BLR-untreated group. SNP did not improve the other indices of mechanical function (table 2).

Glucose Metabolism

Rates for glycolysis, glucose oxidation and proton production in the Fresh Transplant group (optimum conditions for BLR hearts) were very similar to rates for Fresh Hearts from the crystalloid reperfusion model (figure 8). There was a trend towards increased rates of glycolysis in BLR-untreated and treated hearts compared with the Fresh Transplant group. Glucose oxidation rates were again significantly depressed in the BLR-untreated and treated groups and unaffected by SNP. Also noted was a trend towards increased rates of proton production in BLR-untreated and treated hearts compared with the Fresh Transplant group.

Figure 7. Effect of Sodium Nitroprusside on the Recovery of LV Work in the Fresh Transplant Group and in Hearts Subjected to Cardioplegic Arrest, Prolonged Hypothermic Storage and Reperfusion with Blood

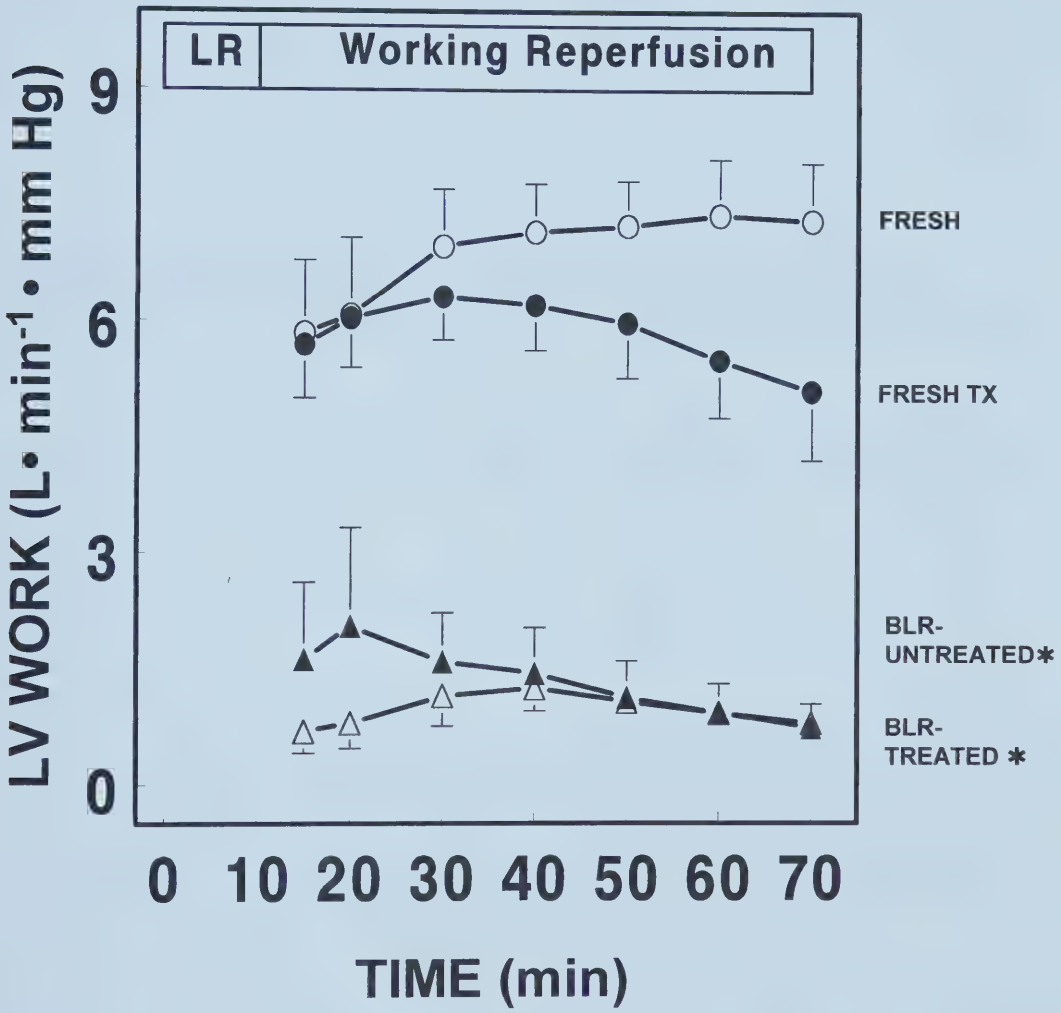


Figure 7. Left ventricular function in working mode reperfusion (**W** phase). Data are shown for Fresh Hearts (non-stored, open circles, n=6), Fresh Transplant hearts (nonstored, blood reperfused, black circles, n=6), BLR-untreated hearts (5 hr storage, blood reperfused, black triangles, n=5) and BLR-treated hearts (5 hr storage, blood reperfused, open triangles n=6). **LR** represents the Langendorff reperfusion phase ($\mathbf{L_2}$ phase) for all hearts. Data are shown as the mean ± the standard error of the mean. ✱ There were no significant differences in LV work between the BLR-untreated and BLR-treated groups.

Table 2. Effect of Sodium Nitroprusside on Recovery of Myocardial Function in the Fresh Transplant Group and in Hearts Subjected to Cardioplegic Arrest, Prolonged Hypothermic Storage and Reperfusion with Blood

Group	n	CO ($mL \cdot min^{-1}$)	AF ($mL \cdot min^{-1}$)	CF ($mL \cdot min^{-1}$)	CVC ($mL \cdot min^{-1} \cdot mmHg^{-1}$)
Fresh Tx [a]	6	47.8 ± 6.2	30.7 ± 4.3	17.2 ± 2.7	0.19 ± 0.03
BLR-untreated	5	8.60 ± 3.1	1.60 ± 1.6	7.00 ± 2.8	0.09 ± 0.03
BLR-treated	6	11.5 ± 2.5	0.30 ± 0.3	11.2 ± 2.5	0.17 ± 0.06

Data are shown as the mean ± the standard error of the mean of the given number of observations (n) at the end of 60 min of working reperfusion. [a] Denotes the Fresh Transplant group.

CO = cardiac output; AF = aortic flow; CF = coronary flow; CVC = coronary vascular conductance; SNP = sodium nitroprusside (200μM)

Figure 8. Effect of Sodium Nitroprusside on Myocardial Glucose Metabolism and Proton Production in the Fresh Transplant Group and in Hearts Subjected to Cardioplegic Arrest, Prolonged Hypothermic Storage and Reperfusion with Blood

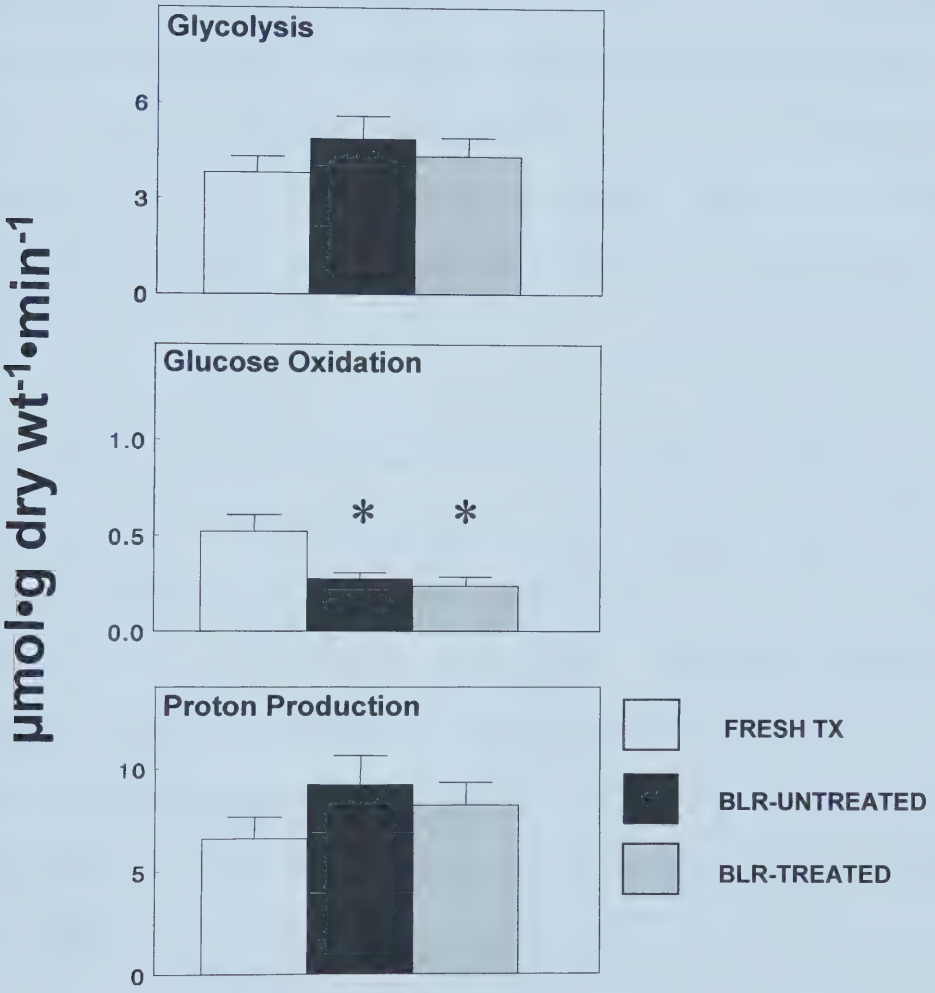


Figure 8. Myocardial glucose metabolism (µmol•g dry wt^{-1}•min^{-1}) in transplanted rat hearts after 1 hr of blood reperfusion. All results were measured and averaged over 60 min of working mode reperfusion. Data are shown for Fresh Transplant hearts (non-stored, blood reperfused, n=6), BLR-untreated hearts (5 hr storage, blood reperfused, n=5) and BLR-treated hearts (5 hr storage, blood reperfused, n=6). Data are shown as the mean ± the standard error of the mean. * Glucose oxidation was significantly depressed in both the BLR-untreated and treated groups compared to the Fresh Transplant group ($p<.05$). There were no significant differences in glycolysis, glucose oxidation and proton production between BLR-untreated and treated groups.

Effect of SNP on Myocardial cGMP Content

The presence of SNP at the time of cardioplegic arrest, storage (5 hr) and at the onset of blood reperfusion resulted in a 7-fold increase in cGMP content (measured immediately prior to blood reperfusion) in BLR-treated hearts compared with the BLR-untreated group (figure 9). This was a statistically significant increase in cGMP content ($p<0.05$). In fact cGMP levels were elevated to a point comparable with the Fresh Transplant group.

This augmentation of cGMP content was not maintained throughout the blood reperfusion phase of the experimental protocol (figure 10). After 1 hr of blood reperfusion cGMP levels were depressed in all groups. This level in the Fresh Transplant group was 0.39 pmol•mg protein^{-1} which was significantly lower than that reported for Fresh Hearts reperfused with crystalloid (1.00 pmol•mg protein^{-1}).[132] There was a trend towards decreased cGMP content in BLR-untreated and treated hearts compared with the Fresh Transplant group after 1 hr blood reperfusion. This decreased was not significantly affected by SNP.

Figure 9. Effect of Sodium Nitroprusside on Myocardial cGMP Content Prior to Blood Reperfusion

Figure 9. cGMP content (pmol • mg protein $^{-1}$) in rat hearts prior to transplantation and blood reperfusion. Data are shown for Fresh Transplant hearts (non-stored, n=4), BLR- untreated hearts (5 hr storage, n=4) and BLR-treated hearts (5 hr storage, n=4). Data are shown as the mean ± the standard error of the mean. * There was a significant decrease in cGMP content in BLR-untreated hearts compared with BLR-treated hearts ($p<0.05$).

Figure 10. Effect of Sodium Nitroprusside on Myocardial cGMP Content after Transplantation and Blood Reperfusion

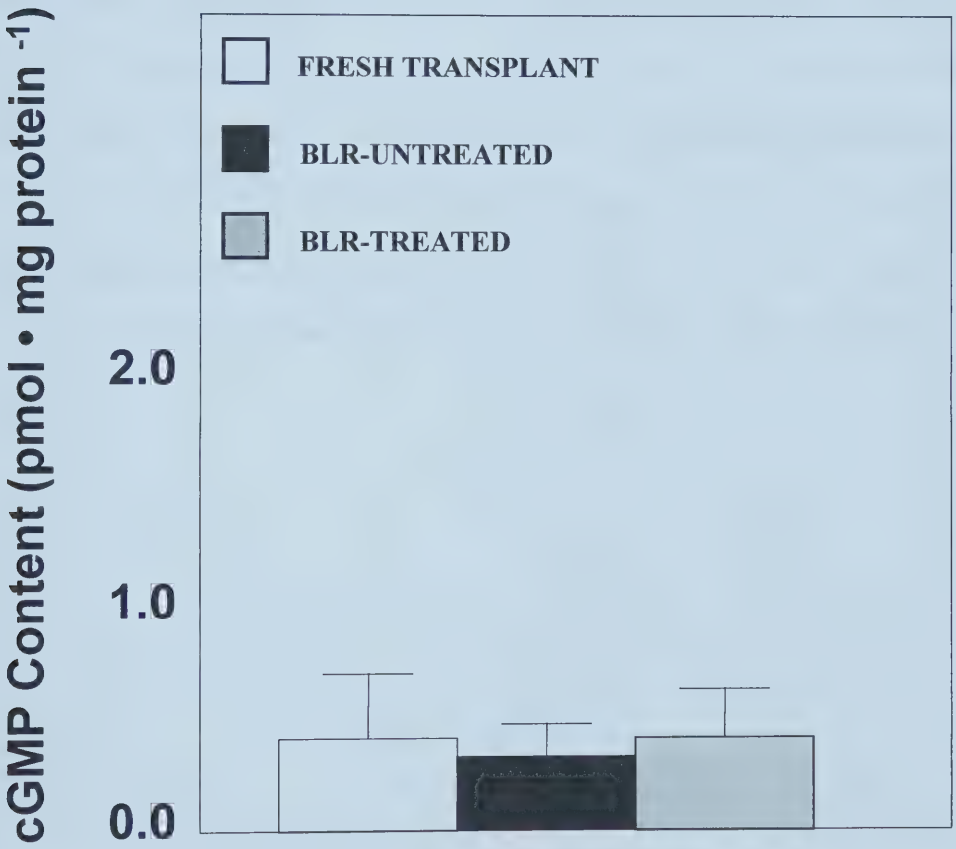


Figure 10. cGMP content (pmol • mg protein $^{-1}$) in the Fresh Transplant group and in hearts subjected to cardioplegic arrest, prolonged hypothermic storage and blood reperfusion. Data are shown for Fresh Transplant hearts (non-stored, n=4), BLR- untreated hearts (5 hr storage, n=4) and BLR-treated hearts (5 hr storage, n=4). Data are shown as the mean ± the standard error of the mean.

Effect of SNP on Myocardial Myeloperoxidase Activity after Reperfusion with Blood

Levels of myeloperoxidase activity have been reported as low as 1-2 mU•mg protein^{-1} for normal hearts.[133] The Fresh Transplant group (optimum blood reperfusion conditions) had an MPO activity of 5.18 ± 0.53 mU•mg protein^{-1} (figure 11). The modestly elevated levels of tissue activity are not surprising considering that these hearts are subjected to approximately 1.5 hrs of ischemia during the transplantation procedure. Myeloperoxidase activity was significantly elevated in the BLR-untreated and treated groups (>100%) compared with the Fresh Transplant group and was essentially unaffected by SNP.

Figure 11. Effect of Sodium Nitroprusside on Myocardial Myeloperoxidase Activity after Reperfusion with Blood

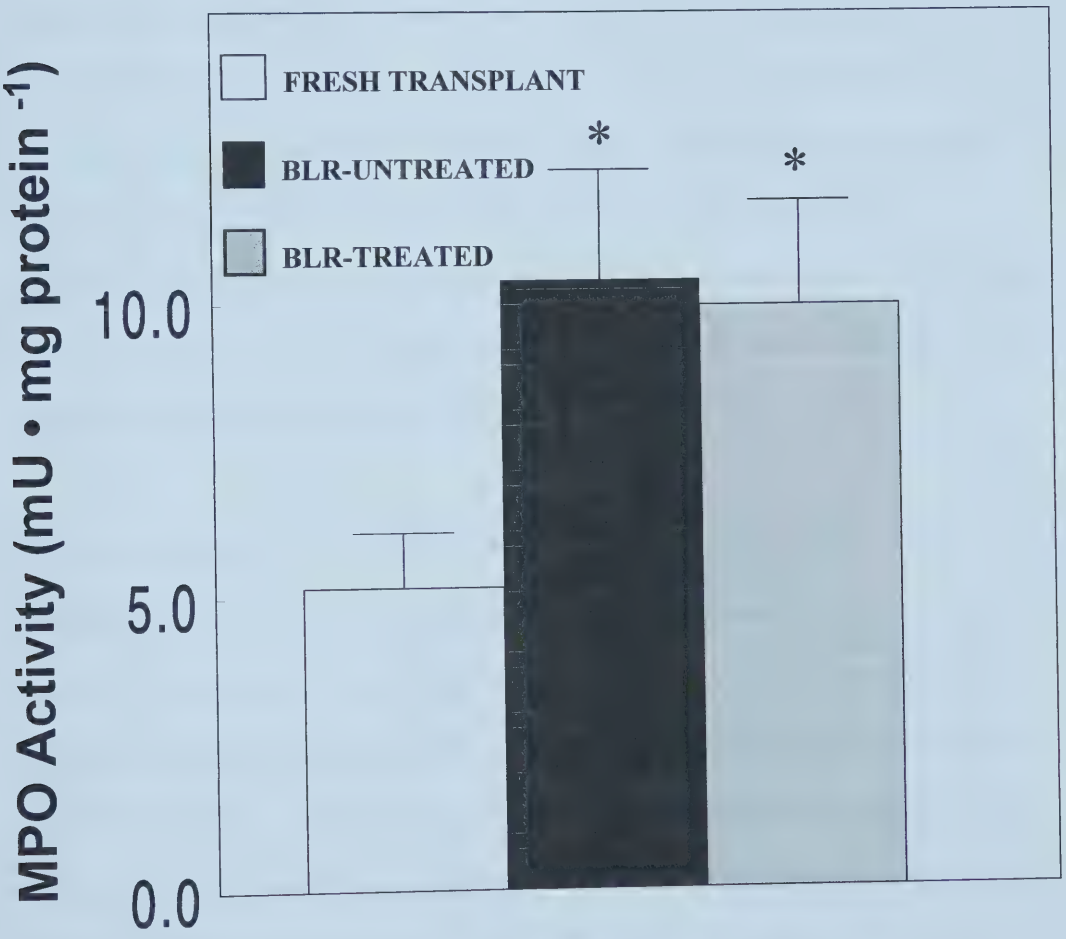


Figure 11. Myeloperoxidase activity (mU • mg protein^{-1}) in rat hearts after 1 hr of blood reperfusion. Data are shown for Fresh Transplant hearts (nonstored, blood reperfused, n=6), BLR-untreated hearts (5 hr storage, blood reperfused, n=5) and BLR- treated hearts (5 hr storage, blood reperfused, n=6). Data are shown as the mean ± the standard error of the mean. * There was a significantly lower amount of myeloperoxidase activity in the Fresh Transplant group compared to the BLR-untreated and treated groups of hearts ($p<.05$).

DISCUSSION

The maximum "safe" ischemia time for cardiac allografts is somewhere between 4-6 hours, after which there is a direct correlation between ischemia time and patient morbidity and mortality. Strategies successfully prolonging acceptable organ ischemia times would overcome geographical barriers to cardiac transplantation in addition to minimising damage secondary to storage and reperfusion. There are various pharmacological strategies currently under investigation, with the objective of preventing injury from ischemia and reperfusion. This study explored the potential for SNP-induced cardioprotection after global ischemia and reperfusion.

The enhanced recovery of mechanical function in CR-treated hearts subjected to cardioplegic arrest, prolonged hypothermic storage and reperfusion with crystalloid was confirmed. Two groups of experiments were performed: one receiving *in vitro (n=12)* crystalloid reperfusion (CR) and the other *in vivo (n=12)* blood reperfusion (BLR) with the use of a heterotopic rat heart transplantation model. SNP improved recovery of LV function in the CR group (8 hr storage) by 100%. This improvement in post-storage LV function is similar to that reported by Ali *et al.*[66] in 1998. Furthermore this finding suggests that safe ischemia times may be extended by the addition of SNP to standard cardioplegia solutions and that post-storage myocardial stunning may be lessened.

Optimal function in transplanted hearts was determined in the Fresh Transplant group of hearts (6.31±0.56 $L \cdot min^{-1} \cdot mmHg$). This level of function was almost equivalent to that

observed in the Fresh Heart group. This suggests that the transplantation procedure itself caused minimal damage to these hearts. Recovery of LV function in the BLR-untreated group was poor (23% of optimal function). Despite an increased cGMP content of hearts in the BLR-treated group (2.02±0.33 vs 0.28±0.04 pmol·mg protein^{-1}, $p<.05$) compared with the BLR-untreated group, an enhanced recovery of LV function was not observed. Suprisingly, a recovery to only 22% of optimal function was observed in the BLR-treated group of hearts. There are several possible explanations for this lack of functional recovery. Methodological factors such as the timing and dosage of SNP administration to stored hearts need to be considered. Another possibility includes the inability of SNP to inhibit myocardial neutrophil infiltration at the time of reperfusion. Myeloperoxidase (marker for neutrophil infiltration, Bradley *et al*[35]) was elevated in both BLR groups compared with the Fresh Transplant group ($p<.05$). Neutrophil infiltration was not affected by SNP.

Whereas SNP protected hearts subjected to cardioplegic arrest, hypothermic storage and crystalloid reperfusion, this NO/cGMP-mediated cardioprotection was not observed in blood reperfused hearts. A deleterious role for neutrophils, platelets and other cellular elements in the lack of myocardial protection by SNP is suggested.

Experimental Models

The rat heart model of global ischemia is well-proven for studies of cardiac IR injury.[66] Left ventricular function, a major predictor of patient survival after cardiac transplantation, is ultimately the most important outcome measure in studies of global ischemia. LV function is reliably assessed on the isolated heart perfusion apparatus. This model simulates physiologic conditions by providing an appropriate energy demand and is unlike nonworking isolated models (Langendorff) in which hearts do not perform external work.[66] Flow meters and pressure transducers allow the measurement of cardiac output and developed pressure from which an index of LV function can be calculated. All hearts are permitted to beat spontaneously at 300 beats•min^{-1} or in the event of bradycardia are paced at this rate in an attempt to standardise energy demand between the various groups of stored hearts. Energy substrates are provided in the form of a Krebs-Henseleit solution containing glucose (11 mM) and palmitate (1.2 mM). The high fat concentration is designed to simulate post myocardial ischemia conditions where circulating free-fatty acid concentrations are high and the heart derives 90% of its ATP requirements from fatty acid oxidation. High rates of fatty acid oxidation are also known to contribute to post-ischemic injury.[134]

Crystalloid Reperfusion

Recovery of Left Ventricular Function

There has been a recent interest in NO within the myocardial protection literature. NO is a regulator of vascular and endothelial integrity and has an established beneficial role in the biochemistry of postischemic reperfusion injury.[2-12] This, along with NO's proven efficacy and safety in the diagnosis and treatment of cardiovascular disease, is responsible for the interest. In the area of cardiac transplantation, NO protects hearts and improves the recovery of LV function in a crystalloid model of prolonged storage (8hr) and reperfusion.[66]

The ability of SNP (NO donor) to protect stored hearts (8 hrs at 4^0C) reperfused with crystalloid was confirmed by this study. Recovery of left ventricular function in CR hearts was improved by the presence of SNP (200μM) throughout all phases of the perfusion protocol. Hearts in the CR-treated group recovered to 70% of optimal function, which was a statistically significant improvement when compared with the CR-untreated group ($p<.05$). There are several mechanisms potentially responsible for this improved recovery of function in CR-treated hearts. NO is believed to protect hearts primarily through a cGMP-mediated mechanism. This is substantiated not only by the ability of the guanylyl cyclase inhibitor ODQ (1H-[1,2,4]oxadiozolo[4,3-a]quinoxalin-1-one), to counteract the beneficial effects of NO, but by the potentiation of NO-induced cardioprotection by zaprinast (phosphodiesterase inhibitor).[132] ODQ's ability to negate the cardioprotective effects of SNP in CR hearts suggests that soluble guanylyl cyclase

(sGC) activation along with other components of NO biosignaling downstream of this are responsible for the SNP-mediated cardioprotection (refer to figure 1).[66] Zaprinast, by inhibiting the breakdown of cGMP, lends further support to this theory.

The precise mechanism by which cGMP protects ischemically damaged myocardium is largely unknown. It probably directly or indirectly alters Ca^{2+} homeostasis (influx via membrane ion channels and release via the sarcoplasmic reticulum) within the myocyte during the critical period of reperfusion. This in turn leads to enhanced myocardial relaxation and prevention of muscular contracture. It is also possible that cGMP positively influences myocardial glucose metabolism by improving the coupling between glycolysis and glucose oxidation. Whether it is through the inhibition of glucose uptake into the myocyte, stimulation of the rate limiting enzymes of glucose oxidation or through the inhibition of fatty acid metabolism, there is a shift away from glycolysis and towards glucose oxidation. This secondarily results in an attenuation of intracellular lactate and proton accumulation and therefore decreased influx of Ca^{2+} within the myocyte.[134-135] As discussed earlier, Ca^{2+} accumulation leads to apoptosis and cell death.

Previous work by Ali *et al.*[66] suggests that the timing of NO/cGMP augmentation is important. They looked specifically at the dependence of functional recovery on the timing and concentration of SNP administration. Pharmacological intervention was found to be most effective at the time of early reperfusion (post-storage). Functional recovery of the LV however, was maximised with SNP present throughout all phases of the perfusion protocol. There was also a concentration dependence of the recovery of LV

function on SNP. Cardioprotection was demonstrated with concentrations of SNP of 50μM, although maximal benefit was achieved at SNP concentrations of 200μM. In the current study SNP (200μM) was provided throughout all phases of the experimental protocol in order to achieve maximal benefits from NO augmentation and to ensure adequate levels of cGMP at the critical moment of reperfusion.

A deficiency in myocardial NO biosignaling has been shown to occur after cardioplegia and storage, resulting in low levels of intracellular cGMP. The mechanism by which cardioplegia and storage result in a decrease in cGMP levels is unknown. Rach *et al.*[132] suggest that it may be secondary to acidosis-induced inhibition of NOS activity, reduced availability of GTP for cGMP generation or enhanced inactivation of endogenous NO by superoxide anion leading to the accumulation of peroxynitrite within the myocyte. All of these events are known to have detrimental effects on myocardial tissue recovering from an ischemic insult. The final common pathway involves low intramyocardial levels of cGMP and the accumulation of OFR's within the myocyte. The end result being the alteration in Ca^{2+} homeostasis, glucose metabolism and lipid membrane stability in a fashion that is harmful to the myocardium.

Glucose Metabolism in Stored Hearts Reperfused with Crystalloid

SNP has been shown to significantly enhance the functional recovery of CR-hearts through a suggested NO/cGMP-mediated mechanism.[66] As discussed previously, cGMP is thought to favourably influence myocardial energy substrate metabolism by improving the coupling between glycolysis and glucose oxidation. The results from the present

study suggest a trend towards increased glucose oxidation rates in CR-treated hearts when compared to the CR-untreated group (figure 6).

A number of clinical and experimental studies demonstrate that the shifting of energy substrate preference away from fatty acid oxidation and towards glucose oxidation can protect hearts during and after periods of ischemia.[134] Several pharmacological agents (e.g., trimetazidine, ranolazine and dichloroacetate) that stimulate glucose oxidation have been shown to improve the recovery of mechanical function following myocardial ischemia.[137] Glucose oxidation is the more efficient method of energy production by the heart and any strategy to augment glucose oxidation will result in significant benefits at the time of reperfusion (decreased Na^+/H^+ and Na^+/Ca^{2+} exchange). Fatty acid oxidation and glycolysis are less desirable methods of ATP production in that they result in the accumulation of lactate and protons within the myocyte (glycolysis) and consume larger amounts of oxygen per ATP produced (fatty acid oxidation). The increase in H^+ load contributes to a well-described Ca^{2+} overload in the ischemic heart. This is secondary to an increase in activity of the Na^+/H^+ exchanger coupled to Na^+/Ca^{2+} exchange. Inhibition of these ion exchangers in the post-ischemic heart (increased glucose oxidation) results in a significant improvement in cardiac function and efficiency.[137]

During reperfusion, a rapid recovery of myocardial energy production is essential for the complete recovery of contractile function. The tendency is for fatty acid oxidation to dominate as the source of ATP production, secondary to high serum levels of free fatty acids. This will result in poor cardiac efficiency unless glucose oxidation is stimulated at

this critical point of muscle reperfusion. The metabolic data from the present study suggest that the shifting of energy substrate preference by the heart (towards glucose oxidation) is at least in part responsible for the enhanced recovery of mechanical function in the CR-treated group of hearts.

Blood Reperfusion

Recovery of Left Ventricular Function

The blood reperfusion model (heterotopic transplantation) was designed to simulate the conditions present during clinical cardiac transplantation. An 8 hr storage period for hearts was initially chosen based on the functional impairment observed in the crystalloid model. This was subsequently decreased to 5 hrs, as organs stored longer than this were found to be damaged beyond the point of functional recovery ("stone hearts"). The shorter storage interval resulted in an appropriate level of damage for which hearts could be expected to recover LV function with the addition of an efficacious pharmacological agent (30% recovery of the optimal function measured in the Fresh Transplant group). SNP was administered in a clinically relevant fashion at the time of cardioplegic arrest, during storage and as a "terminal hot shot" prior to blood reperfusion. This model has the advantage of avoiding the systemic administration and deleterious hemodynamic effects of SNP.

As discussed above, SNP was found to be cardioprotective in crystalloid reperfused hearts, but failed to enhance the recovery of BLR-treated hearts. Hearts in the BLR-

treated group demonstrated a very poor recovery of LV function (22%) which was not significantly different from the BLR-untreated group. This lack of cardioprotection by NO in BLR hearts contradicts results from Pinsky *et al.*[119] and other groups demonstrating cardioprotection by NO in the blood reperfusion environment.[116-121] Of note however, is that most blood reperfusion studies showing cardioprotection by NO are based on short-term (1-2 hrs) models of global ischemia.[117-18,120-21] Although this is a good starting point to investigate the cardioprotective potential of NO after global ischemia, an ischemia time less than 4 hrs is not clinically relevant and not necessarily indicative of results in a long-term (> 4 hrs) storage model of blood reperfusion.

There are two previous studies looking at NO-mediated protection after prolonged hypothermic storage.[116,119] Pinsky *et al.*[119] suggest that NO/cGMP augmentation enhances graft survival after 12 hr storage and blood reperfusion (heterotopic rat heart transplantation model). Unfortunately, they looked only at qualitative outcome measures (presence of contractions, EKG depolarisations and graft color) and failed to objectively assess mechanical function in these hearts. Graft survival is important, but it is essential to demonstrate that these organs will work effectively as pumps after prolonged periods of global ischemia. This has not been achieved thus far in studies of NO-mediated cardioprotection after prolonged hypothermic storage. Similar problems are evident in the study by Oz *et al.*[116] where outcome measurements (ability to wean animals from cardiopulmonary bypass as a positive outcome) and lack of follow-up work weaken the evidence for NO-mediated cardioprotection with blood reperfusion after prolonged storage.

NO and cGMP content

The data from the cGMP assays in this model suggest that the answer for the lack of cardioprotection provided by NO may lie downstream from the conversion of GTP to cGMP by guanylyl cyclase. Cyclic GMP content was significantly elevated in BLR-treated hearts compared to the BLR-untreated group (7-fold increase at the time of blood reperfusion). This finding, along with the excellent recovery of LV function in the Fresh Transplant group, confirms the validity of the blood reperfusion model.

It is important to note that the augmentation of cGMP content was not maintained throughout the entire blood reperfusion phase of the experimental protocol (figure 10). After 1 hr of blood reperfusion cGMP levels were depressed in all groups. The level measured in the BLR-treated group was $0.364 \pm .06$ pmol•mg protein^{-1} which was significantly lower than that reported for CR-treated hearts prior to working mode perfusion ($2.07 \pm .33$ pmol•mg protein^{-1}). The failure to maintain high cGMP levels throughout the blood reperfusion period could very well be responsible for the lack of cardioprotection by NO in this heterotopic transplantation model. The more likely explanation is this decline in cGMP levels (during blood reperfusion) is a normal phenomenon due to the nucleotide's extremely short half-life. The high levels of cGMP are certainly most crucial at the onset of reperfusion and the results from this study suggest alternative etiologies for myocardial damage in the blood reperfusion environment not seen in the crystalloid model. Unfortunately, cGMP levels 1 hr after crystalloid reperfusion are not available in this model of prolonged ischemia.

The potential inactivation of NO by superoxide radicals or the heme moiety of hemoglobin not seen in the crystalloid model of reperfusion has been suggested. NO administration to biological systems is difficult, yet there are several strategies available to overcome this problem. In this model NO was administered close to the site of ischemic damage and systemic exposure was avoided as previously described. The elevated cGMP levels downstream of NO suggest that the route of administration was successful in the current investigation. Studies of short-term global ischemia (blood reperfusion) demonstrating cardioprotection by NO, indicate that the bioavailability of NO is not significantly affected by the presence of free radicals or hemoglobin. Nevertheless it is possible that a proportion of available NO at the time of reperfusion was inactivated by pre-formed superoxide radicals to form toxic peroxynitrite ions ($ONOO^-$). The deleterious role of peroxynitrite in IR injury has been discussed extensively in this paper.[48,70]

NO and Myeloperoxidase activity

Myeloperoxidase activity was significantly elevated in the BLR-treated and untreated groups (>100%) compared with the Fresh Transplant group and was essentially unaffected by NO. These results suggest a harmful role for neutrophils in ischemia-reperfusion injury. Although the pathogenesis of reperfusion injury is multifactorial, it is well established that neutrophils contribute greatly to this injury after ischemia.[77-82] The role of NO in neutrophil-mediated damage is controversial. Some studies (not uncontested) have demonstrated the ability of NO to inhibit neutrophil chemotaxis, activation and adherence to the endothelium, all of which play a key role in reperfusion

injury.[104-5] To date, all studies dealing with this issue have been done in short term global ischemia models of blood reperfusion.

The mechanism by which NO inhibits neutrophil-mediated reperfusion injury is largely unknown, however several mechanisms have been postulated. [12,138-40] NO is known to inactivate superoxide radicals and directly inhibit their production from neutrophils. Secondly, NO is believed to down-regulate the expression of adhesion molecules on the neutrophil (CD11/CD18) and intercellular adhesion molecule-I (ICAM-I) on endothelial cells. Further investigation is required to further define these actions of NO. The present study demonstrates that the infiltration/activation was unaffected by NO. This could be due to the severity of ischemia and the conditions of reperfusion which were designed in a clinically relevant fashion.

Glucose Metabolism in Stored Hearts Reperfused with Blood

In the blood reperfusion situation (figure 8) glucose oxidation was significantly depressed in both BLR-treated and BLR-untreated hearts compared with the Fresh Transplant group. This secondarily resulted in an increased proton production in these hearts. Whether this explains the lack of cardioprotection by NO in the blood reperfusion environment is unknown (refer to previous discussion of glucose metabolism from crystalloid reperfusion discussion). It is most likely the decreased efficiency in energy production is secondary to the poor recovery of mechanical function in these hearts.

Conclusion

SNP protects hearts subjected to cardioplegic arrest, prolonged hypothermic storage and reperfusion with crystalloid. This cardioprotection is believed to occur primarily through a NO/cGMP-mediated mechanism. This project evaluated SNP's potential to attenuate IR injury in the more clinically relevant situation of blood reperfusion. SNP was found to increase cGMP content in BLR hearts at the time of reperfusion but this did not translate into a benefit in terms of functional recovery. We realize that the etiology of myocardial IR injury is multifactorial and that the potential reasons for this lack of cardioprotection are multiple.

Methodological factors must be considered including the timing and dosage of SNP administration. This could significantly influence the production of $ONOO^-$, a known cardiotoxic byproduct of NO metabolism. Unfortunately, we cannot simply extrapolate results from crystalloid models of reperfusion to the blood reperfusion environment. Neutrophils, platelets, hemoglobin and various other bloodborne elements all play a significant role in myocardial reperfusion injury. It is NO's effect on these bloodborne constituents and the resultant impact on myocardial IR injury that is unknown.

Blood reperfusion models after short periods of ischemia demonstrate promise for NO as myocardial protective agent. Time will solve the problem of IR injury in hearts subjected to prolonged periods of hypothermic arrest. Whether NO will contribute significantly in this area remains to be seen. Further investigations are needed using a variety of NO

donors (e.g., L-arginine), as well as other methods of augmenting cGMP presence within the myocyte (e.g., lipopolysaccharide, phosphodiesterase inhibitors). As in many areas of medicine, the answer may lie with combination therapy as experimental work needs to incorporate free-radical scavengers, neutrophil inhibitors (e.g., adenosine, antioxidants, antiproteases and neutrophil filters) and antiplatelet agents.

This work would also support the concept of a simpler model of *in vitro* blood reperfusion on the isolated heart perfusion apparatus. After rigorous *in vitro* study, promising protocols could be advanced for further investigation with a more complex model of animal transplantation.

The future of human cardiac transplantation is bright with the current expected 10 year survival rates in excess of 65%. Studies of myocardial reperfusion injury and the development of novel pharmacologic strategies to prolong the "safe" ischemia time and prevent damage to cardiac allografts have many potential clinical benefits. Along with a more efficient use of available organs, improved graft function will decrease recipient morbidity and mortality after cardiac transplantation.

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